AMINO ACID EFFECTS IN THE FELINE CENTRAL NERVOUS SYSTEM

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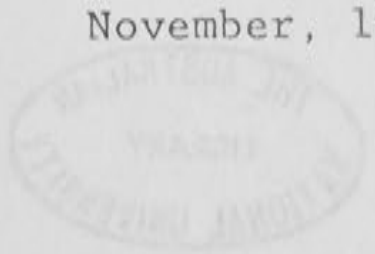

Due to the complex nature of the neuropharmacological experiments described in this thesis, collaboration with Professor D.R. Curtis (Chapters 3 and 7) and other members of the Department of Pharmacology was necessary. Dr J.D. Leah participated in the experiments described in Chapters 3,4,5 and 7, Dr J.C. Bornstein in those described in Chapter 7 and Mr R. Malik in the experiments of Chapter 6.

(Martin James Peet).

During the tenure of my Australian National University Scholarship the following papers have been published or accepted for publication:

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Curtis, D.R., Lodge, D., Bornstein, J.C. and Peet, M.J. Selective effects of (-)-baclofen on spinal synaptic transmission in the cat. Exp. Brain Res. 42 (1981) 158-170.

Curtis, D.R., Lodge, D., Bornstein, J.C., Peet, M.J. and Leah, J.D. The dual effects of GABA and related amino acids on the electrical threshold of ventral horn group Ia afferent terminations in the cat. Exp. Brain Res., (1982) .

Krogsgaard-Larsen, P., Hansen, J.J., Lauridsen, J., Peet, M.J., Leah, J.D. and Curtis, D.R. Glutamic acid agonists. Stereochemical and conformational studies of DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and related compounds. Neurosci. Lett. 31 (1982) 313-317.

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Peet, M.J., Malik, R. and Curtis, D.R. Postexcitatory depression of neuronal firing by acidic amino acids and acetylcholine in the cat spinal cord. Brain Research (1983) .

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SUMMARY

In this thesis, the use of a series of recently synthesized amino acid antagonists provides support for a previous proposal that, in addition to receptors for glutamate and aspartate, there exists a separate receptor for kainate on interneurones in the feline spinal cord. As with other investigations of this kind, the degree of difference in the antagonism of responses to L-glutamate and L-aspartate was less than with their potent analogues DL-quisqualate and NMDA, respectively. Thus, compounds such as 2-amino-5-phosphonovalerate and 2-amino-7-phosphonoheptanoate selectively antagonized responses to NMDA with minimal effects on those by DL-quisqualate and DL-kainate. In contrast, compounds such as γ-D-glutamylglycine, β-D-aspartyl-β-alanine and DL-cis-2,3-piperidine dicarboxylate reduced excitations by NMDA and DL-kainate but were less effective against those by DL-quisqualate.

An investigation of the effects of these antagonists on the synaptic excitation of identified dorsal horn interneurones, showed that those compounds capable of antagonizing the excitation by microelectrophoretic L-aspartate and NMDA, also reduced the polysynaptic activation of these cells. Since none of these compounds affected monosynaptic excitation evoked in the same interneurones, or the ventral root-evoked excitation of Renshaw cells, these results provide additional evidence for the involvement of L-aspartate as a transmitter of feline dorsal horn interneurones.

The stereoisomers of the glutamate analogue, AMPA, and some related compounds were used in an investigation of the stereochemical and conformational requirements necessary for activation of central glutamate receptors. Although both D- and L-AMPA evoked GDEE- (but not 2APV-) sensitive excitation of dorsal horn interneurones, stereochemical aspects were obviously also important in the action of these compounds since L-AMPA was approximately four to six times more potent than D-AMPA. The potency of AMPA, and the related compound ATPA, may in part be due to the steric effect of the methyl group of AMPA and the bulky tertbutyl group of ATPA on the amino acid side chain of these compounds.

The mechanism responsible for the depression of background firing seen in some dorsal horn interneurones and Renshaw cells following intense excitation evoked by microelectrophoretically-administered compounds appears to be directly related to the amount of firing of the cell. A recent investigation of this phenomenon suggested that the postexcitatory depression seen with for example, L-glutamate and L-aspartate, was due to the in vivo decarboxylation of these compounds to GABA and ß-alanine, respectively. No evidence to support such a proposal was obtained in this present study.

Finally, the biphasic (increase/decrease) effect of GABA (reported in a previous study from this laboratory) on the threshold of single Ia afferent terminations in the ventral horn motor nuclei of the feline spinal cord was investigated. While the decrease in threshold by GABA appeared to involve membrane conductance changes, the increase appeared to be indirect and due to the Na^+-coupled uptake of this amino acid.

GENERAL INTRODUCTION

This thesis is concerned with a microelectrophoretic determination of the pharmacology of amino acid receptors and effects produced by amino acids in the feline spinal cord.

The first part of this study involves the use of a series of newly synthesized antagonists to confirm that at least three classes of receptor exist for excitant amino acids on spinal interneurones and Renshaw cells and reports an investigation of the conformational and stereochemical requirements necessary for activation of the central glutamate class of receptor. In addition this section also considers the mechanism(s) underlying postexcitatory depression, a phenomenon related to the excitation of central neurones by intense synaptic or chemical excitation.

A previous investigation in this laboratory of the effects of GABA on the excitability of single primary afferent terminals in the ventral horn of the feline lumbar spinal cord showed that this amino acid generally increased excitability, an effect interpreted as depolarization of the terminal portion of the afferent fibre. However, biphasic (increase/decrease) alterations in excitability were also recorded during microelectrophoretic ejection of GABA and the second part of this thesis investigates the mechanism(s) responsible for these decreases in excitability.

Thus this review will consider the literature implicating amino acids in synaptic transmission. With regard to the excitant amino acids most emphasis will be placed on those

compounds most likely to be excitatory transmitters i.e. L-aspartate and L-glutamate, although it will be necessary to consider other analogues during the review of the literature on receptor classification and receptor structural requirements. Regarding the involvement of GABA in synaptic transmission, evidence describing both pre- and postsynaptic inhibitory effects of this compound will be considered.

Evidence concerning the involvement of amino acids in central synaptic transmission includes information about:-

(a) Synthesis and storage within, and release from, presynaptic terminals.

(b) Receptors for amino acids and their rôle in synaptically-induced postsynaptic events.

(c) Interaction with postsynaptic receptors and the underlying ionic mechanism(s) of action.

(d) Termination of the action of amino acids.

(a) Synthesis and storage within, and release from, presynaptic terminals

Synthesis and storage

Excitant amino acids

Concern has been expressed (Curtis, 1979) regarding the significance of biochemical studies related to L-glutamate and L-aspartate as transmitters because of their concurrent involvement in general metabolism. For instance, L-glutamate and L-aspartate are active in general deamination-reamination reactions and L-glutamate is also involved in the synthesis of

certain special products such as glutathione, carbamylglutamate in the ornithine cycle, and glutamine. A major metabolic rôle for L-aspartate is in the conversion of citrulline to arginine, again a compound important in the ornithine cycle.

Relatively little is known specifically about the in vivo metabolism or synthesis of L-aspartate or L-glutamate in presynaptic endings. L-apartate can be formed in cerebral tissue in the presence of the enzyme aspartate transaminase by transamination of L-glutamate with oxaloacetate to yield L-aspartate and α-ketoglutarate. Mammalian cortical synaptosomes (de Belleroche and Bradford, 1972) have been shown to rapidly oxidise [U-^{14}C]glucose to L-glutamate and L-aspartate, which upon electrical or chemical stimulation in the presence of calcium are discharged into the medium.

An important precursor in the synthesis of L-glutamate is glutamine and in brain tissue there is an extensive conversion of glutamine into glutamate (Van den Berg, Kržalic, Mela and Waelsch, 1969). Nerve terminals apparently lack the glutamine synthesizing enzyme glutamine synthetase (Salganicoff and De Robertis, 1965), but it is present in glial cells. These cells are able to use many precursors (acetate, pyruvate and amino acids including synaptically-released L-glutamate) to produce glutamine. It is envisaged that the glutamine is then released into the extracellular space where it is taken up by the terminals (Ramaharobandro, Borg, Mandel and Mark, 1982). Nerve terminals have a high glutaminase activity (Bradford and Ward, 1976) and at CSF levels (0.5 mM) glutamine (in the presence of 10 mM glucose) is readily used as a substrate by

rat synaptosomes and contributes at least 50% of the carbon to L-glutamate. Thus control of L-glutamate synthesis could be either by increasing glutamine uptake or by control of activation of the enzyme glutaminase, or both. End product inhibition is presumably important since in the hippocampal slice (Cotman and Hamberger, 1978) L-glutamate synthesis increases upon its release in response to depolarization (see also Yoneda, Roberts and Dietz, 1982).

Specific nucleus or pathway lesions have been found to be useful in determining the association of an amino acid with transmission at related presynaptic terminals since as a consequence of terminal degeneration, there may be alterations in amino acid concentration which are detectable by neurochemical analysis.

Reduction in amino acid concentration was found in the fascia dentata of rat hippocampal tissue after unilateral lesions of the entorhinal cortex (Di Lauro, Schmid and Meek, 1981). Consequent upon the degeneration of perforant path input to this region was the selective decrease in L-aspartate concentration, a reduction that was related to the loss of terminals rather than a decrease in target cells. In contrast Nadler and Smith (1981) have reported that destruction of hippocampal perforant path fibres reduces the L-glutamate content of a crude synaptosomal fraction of the same tissue by about 40% without affecting L-aspartate content.

Other ablation studies showing reduced amino acid concentrations have implicated L-glutamate as the transmitter released from corticothalamic (Bromberg, Penney, Stephenson

and Young, 1981; Fonnum, Storm-Mathisen and Divac, 1981), corticorubral (Bromberg, Penney, Stephenson and Young, 1981) and corticostriatal pathway terminals (Fonnum, Storm-Mathisen and Divac, 1981).

Of particular interest to this present study is the implication of L-glutamate as the transmitter released by some primary afferent terminals in the spinal cord of cats (Graham, Shank, Werman and Aprison, 1967). Since the primary afferents entering the dorsal horn are of heterogeneous origin it is possible that one or more transmitter substances could be involved. For instance substance P, which is an excitant when applied microelectrophoretically to spinal neurones (Henry, Krnjević and Morris, 1975) appears to be synthesized in small dorsal root ganglion cells and transported along dorsal root afferent fibres to terminals located in laminae 1 and 2 in the most dorsal part of the dorsal horn. The evidence for this is based on the accumulation of substance P-reactive material proximal to the ligation of the dorsal root (Hökfelt, Kellerth, Nilsson and Pernon, 1975) and a depletion of substance P in the terminals of fine afferents after sectioning of the peripheral processes of primary sensory neurones (Barbut, Polak and Wall, 1981, Jessell, Tsunoo, Kanazawa and Otsuka, 1979).

Two objections to the proposal that L-glutamate is a transmitter in the spinal cord have been put forward. Firstly, free L-glutamate levels in the dorsal root compared wth the ventral root are not sufficiently different to support a transmitter function in the dorsal root. Free L-glutamate is higher in the dorsal root than in the ventral root but only

from 1.3-2.3 times greater (Johnson, 1972). Secondly, spinal cord L-glutamate levels do not significantly change (per g tissue) after dorsal root input section (Takahashi and Otsuka, 1975). Although the amount of L-glutamate may be high in nerve terminals this level may be insignificant when compared to that involved in cellular metabolism. It has been suggested (Johnson, 1977) that this approach to measuring L-glutamate concentration changes in terms of µmoles of L-glutamate per g tissue may be inadequate and that a more appropriate method is to measure the free L-glutamate/free amino acid concentration ratio changes following nerve injury or denervation. Thus, in fibres where L-glutamate is not a transmitter candidate, (e.g. ventral root fibres and distal sensory root fibres) nerve injury does not result in a slowing down of L-glutamate formation relative to other free amino acids (Porcellati and Thompson, 1957) while in the dorsal root (Johnson, 1976), injury did result in a fall in the free L-glutamate/total free amino acid concentration ratio. Therefore it is not so much the decrease in µmoles of L-glutamate per g tissue, rather the change in amino acid metabolism, in order, during dorsal root injury, to sacrifice L-glutamate formation in favour of other amino acids.

While free L-glutamate levels in dorsal roots and dorsal root ganglion appear to be similar, L-glutamate concentration is highest in the dorsal horn gray matter where dorsal root fibres terminate. Thus its distribution is in agreement with suggestions (Graham, Shank, Werman and Aprison, 1967) that L-glutamate is the transmitter released by primary afferents

and may be responsible for monosynaptic excitation in the spinal cord (Curtis and Johnston, 1974).

In contrast, L-aspartate concentrations are higher in the ventral gray matter. Ligation of the thoracic aorta of cats (Davidoff, Graham, Shank, Werman and Aprison, 1967) produced a loss of polysynaptic excitatory reflexes and an analysis of the spinal gray matter showed a decline of both L-glutamate and L-aspartate concentrations. However, since the decrease in L-aspartate (but not L-glutamate) correlated to the loss of small neurones, L-aspartate may be a transmitter of polysynaptic excitation (Johnson, 1972, 1977) in the mammalian central nervous system.

γ-Aminobutyric acid (GABA)

The synthesis of GABA in central nervous tissue is intimately related to that of glutamine and L-glutamate. Since the first observations of GABA in vertebrate brain tissue (Roberts and Frankel, 1950) and consideration of its rôle in central synaptic transmission (see review by Elliott and Jasper, 1959), studies have demonstrated the interconversion of these three compounds. For example, when labelled glutamine was injected into the cisterna magna of rats there was rapid metabolism of this compound into L-glutamate, GABA and other amino acids (Berl, Lajtha and Waelsch, 1961), and the interconversion of labelled GABA to glutamine has also been demonstrated (Roberts, Rothstein and Baxter, 1958).

Studies designed to manipulate enzymic activity within the glutamine-L-glutamate-GABA cycle have shown that two enzymes are particularly important in controlling GABA concentrations in central nervous tissue. Inhibition of the activity of the GABA-synthesizing enzyme L-glutamate decarboxylase (GAD) by semicarbazide reduces GABA levels in the brains of rats and mice (Maynert and Kaji, 1962) and the spinal cords of cats (Bell and Anderson, 1972; see also Levy, 1977) leading to convulsions and alterations in long latency spinal inhibition and dorsal root recorded potentials, respectively. Further evidence involving GABA in the latter phenomena, and their relationship to primary afferent depolarization (PAD) and presynaptic inhibition, will be discussed in Section c.

Blockade of the activity of GABA transaminase, by amino oxyacetic acid (AOAA), the enzyme responsible for conversion of GABA to glutamine, produces a steady increase in the GABA concentration in the mouse brain for several hours (Van Gelder, 1966) which results in a substantial decline in glutamine synthesis (Van den Berg, Mathison and Nijenmanting, 1978). The possibility exists, however, that AOAA elevates GABA levels in non nerve tissue (Iadorola and Gale, 1979) although in this same study the increase in GABA levels by n-dipropylacetate appeared to involve nerve terminals. AOAA and hydroxylamine increased GABA levels, and at the same time increased PAD in the frog spinal cord (Davidoff, Grayson and Adair, 1973), but in the cat, AOAA has been reported to have little (Polc and Haefely, 1976) or no (Bell and Anderson,

1972) enhancing effect on PAD or dorsal root potentials (DRP), although an increase in the long duration inhibition of monosynaptic reflexes (Polc, Möhler and Haefely, 1974) has been recorded.

Studies of the intraspinal distribution of some depressant amino acids have been carried out (Johnston, 1968) using extracts of feline lumbar gray matter. In the gray matter GABA was found in higher concentrations dorsally (2.18 µmole/g tissue) than ventrally (1.04 µmole/g tissue). The spinal level of GABA was higher in the gray matter than in the dorsal or ventral roots but lower than in the brain. These results were confirmed by Otsuka and Konishi (1975) who extended these studies to demonstrate that the highest GABA containing region was in the dorsal or dorsolateral region of the dorsal horn. This distribution of GABA correlates with levels of GAD activity recorded in similar tissue (Graham and Aprison, 1969). Compartmentation of the synthesizing and degradating enzymes for GABA has been suggested. Early studies have shown that a large fraction (60% or more) of GAD is located in nerve endings and it could be assumed that most of the GABA in the central nervous system is formed in these endings (but see earlier discussion on the effect of AOAA). These GAD containing terminals contain little or no GABA transaminase and therefore the degradation part of the sequence must occur at a separate site. This location may be glial cells (Balazs and Cremer, 1973) although GABA transaminase has been located in both glia and presynaptic endings (Hyde and Robinson, 1976).

Thus the actual site(s) of GABA synthesis and degradation is still controversial and little information is available on mechanisms responsible for control of GABA levels in central nervous tissue. However, it might be assumed that GABA is capable of controlling its synthesis by GAD (Löscher, 1981) and such a negative feedback mechanism onto the enzyme ornithine-δ-aminotransferase by GABA controls a synaptosomal biosynthetic pathway that is capable of producing L-glutamate and GABA from ornithine (Yoneda, Roberts and Dietz, 1982).

Assuming that GAD containing terminals do actually synthesize GABA (Curtis, 1978a) then there is substantial immunocytochemical evidence on the location of GAD at axo-dendritic, axosomatic and axoaxonic synapses to suggest a transmitter rôle for GABA in the mammalian central nervous system. In the rat spinal cord, for example, there are high levels of GAD reaction product in the dorsal horn laminae I-III, and moderately heavy levels of product in layers IV-VI, VII and X. In the ventral horn GAD reaction product was moderately light and was seen on motoneuronal cell bodies (McLaughlin, Barker, Saito, Roberts and Wu, 1975; Wood. McLaughlin and Vaughan, 1976). GAD immunochemistry has also been used extensively to map GAD-containing terminals in other regions of the mammalian central nervous system including the rat corpus striatum (Ribak, Vaughn and Roberts, 1979), rostro-medial substantia nigra and rostral ventral tegmental area (Walaas and Fonnum, 1980) and the deep cerebellar nuclei (Perez de la Mora, 1981). A combination autoradiographic and biochemical study (Hattori, McGeer, Fibiger and McGeer, 1973)

demonstrated that [^{3}H]GABA uptake into slices of rat substantia nigra occurred primarily into nerve terminals and that electrolytic lesions of the globus pallidus (thought to have connections with the substantia nigra) reduced [^{3}H]GABA uptake and decreased GAD activity.

The nature of the terminals showing GAD reaction product has been closely studied at the electronmicroscopic level. In the rat spinal cord, GAD-containing presynaptic terminals, make symmetrical synaptic contacts with both dendrites and cell bodies of dorsal horn interneurones, particularly within laminae I-III, and of ventral horn motoneurones (McLaughlin, Barber, Saito, Roberts and Wu, 1975). In addition, GAD-containing terminals are presynaptic at axoaxonic synapses upon axon terminals in laminae II and III and upon large axon terminals which are presynaptic to motoneurone somata. Non GAD-containing "postsynaptic terminals" within the substantia gelatinosa degenerate after dorsal root section and may be the terminals of excitatory primary afferents (Barber, Vaughn, Saito, McLaughlin and Roberts, 1978). Axoaxonic synapses have also been described in the cat cuneate nucleus (Walberg, 1965) and in the chronically deafferented frog spinal cord (Glusman, Vasquez and Rudomin, 1976). [^{3}H]GABA can be taken up into boutons in laminae I-III which are presynaptic to other terminals which in turn synapse with dendrites (Ljungdahl and Hökfelt, 1973).

Thus these observations of GAD-containing terminals presynaptic to dendrites and cell bodies in both dorsal and ventral horns supports evidence from other sources that GABA,

released from these terminals, mediates postsynaptic inhibition of spinal interneurones and motoneurones in the mammal. The additional finding of GAD-positive terminals (which probably correspond to the P type terminal in the studies of Conradi, 1969a,b) presynaptic to other axonal terminals in the dorsal horn and motor nuclei is supportive of other data (outlined in Section c) implicating GABA in the production of PAD and ultimately presynaptic inhibition in the mammalian spinal cord.

Release

Excitant amino acids

The use of release criterion to implicate an amino acid in central nervous transmission has been most intensively studied in slice or synaptosome preparations in vitro where transmitter release is evoked by the use of depolarizing techniques such as high potassium concentration or agents such as veratridine. The relevance of such "depolarization" to the release of transmitters by presynaptic impulses may be questioned. However, amino acid release into the perfused central canal of the cat spinal cord has been recorded in vivo in response to a generalised activation of descending spinal tracts (Fagg, Jordan and Webster, 1978).

Using rat olfactory cortex slices it has been proposed that L-aspartate is the transmitter of the lateral olfactory tract fibres (Collins, 1979). When amino acid release from the pial surface of synaptically intact rat olfactory cortex

slices was monitored, both electrical stimulation of the lateral olfactory tract and application of high K^+ concentrations evoked a Ca^{2+}-dependent (and therefore presumably neuronal) release of L-aspartate. In this same study unilateral bulbectomy which leads to degeneration of the lateral olfactory tract fibres was accompanied by a specific loss of tissue L-aspartate which was particularly pronounced at the depth at which these fibres terminated (Collins, 1979). Collins and Probett (1981) have also investigated the protoveratrine-A evoked release of endogenous amino acid transmitter candidates from olfactory cortex of control and bulbectomized rats and found that in support of their previous results, bulbectomy was accompanied by a statistically significant and specific attenuation of drug-evoked L-aspartate release.

Other studies (Baughman and Gilbert, 1980, 1981) used high pressure liquid chromatography techniques to measure the release of endogenous compounds from a tissue slice preparation of the visual cortex of the rat. When release was induced, either by raising the K^+ concentration in the medium or by adding veratridine, of the compounds measured, marked increases in release rate were observed for L-aspartate and L-glutamate.

L-glutamate has also been implicated by release studies as a transmitter in the cerebellum (Levi and Gallo, 1981; Levi, Gordon, Gallo, Wilkin and Balazs, 1982) and the hippocampal perforant pathway (Nadler, White, Vaca, Redburn and Cotman, 1977; White, Nadler, Hamberger, Cotman & Cummins, 1977).

GABA

For similar reasons to those relating to the excitant amino acids, few studies (Obata, 1976; Fagg, Jordon and Webster, 1976) have investigated the in vivo release of GABA in response to stimulation of a putative gabergic pathway and most observations have again centred around the Ca^{2+} dependent (Nadler, White, Vaca and Cotman, 1977) release of GABA from tissue slices or synaptosomes by "electrical stimulation" or elevated K^{+} concentration (see review by Cutler, 1976).

Irrespective of the relevance to the in vivo situation slices of the dentate gyrus are capable of efficiently accumulating and releasing exogenous radiolabelled GABA. The release of GABA from those slices was approximately in proportion to their GABA content although in another study by Bondy, Burks and Harrington (1979) on the ability of different parts of the mouse brain and spinal cord to take up and release transmitter, a lack of relationship between the two components was observed.

In the rat and pigeon central nervous systems the L-glutamate synthesizing and releasing pool are different from those for GABA (Reubi, 1981) and in the hippocampal formation GABA content and release appears related to short axon interneurones (Nadler, White, Vaca and Cotman, 1977). Such an involvement of GABA with intrinsic interneurones could be implied by the finding of a decrease in GAD reaction product after the hypoxic destruction of these cells in the spinal dorsal horn (Miyata and Otsuka, 1972).

Receptors for amino acids and their rôle in synaptically-induced postsynaptic events.

Ligand Binding Studies

Excitant amino acids

Many different regions of the brain have been used to investigate receptors for labelled amino acids including hippocampus (Baudry and Lynch, 1979), striatum (Vincent and McGeer, 1979), cerebral cortex (Fiszer de Plazas and De Robertis, 1976) and cerebellar cortex (Foster and Roberts, 1978; Sharif and Roberts, 1980, 1981) and marked regional differences in the level of binding of L-[^{3}H]glutamate to brain membranes have been observed (Biziére, Thompson and Coyle, 1980).

The binding of L-[^{3}H]-glutamate to cerebellar membranes (Foster and Roberts, 1978) was primarily associated with synaptic membrane fractions, and it was possible to separate the binding of L-[^{3}H]-glutamate into either Na^+-dependent or Na^+-independent, the latter thought to represent binding to the postsynaptic receptor and the former binding to the uptake site (Roberts, 1974: see also Baudry and Lynch, 1979). Other differences between these binding sites also appeared to exist, however, since compounds that were good inhibitors of the high affinity uptake of L-glutamate were poor displacers of specific L-glutamate binding. For example, although L-aspartate and L-glutamate compete for the same carrier (Logan and Snyder, 1972) L-aspartate was 10 times less potent at displacing L-[^{3}H]glutamate binding whereas compounds such as the conformationally-restricted glutamate analogues D- and

L-1-amino-1,3-dicarboxycyclopentane (McLennan and Wheal, 1976) and DL-2-amino-4-phosphonobutyrate (2APB) were potent displacers. DL-Kainate had a weak displacing action and N-methyl-D-aspartate (NMDA) was ineffective.

Ligand binding studies (in support of the microelectrophoretic investigations discussed later) have provided evidence for differences between receptors for L-aspartate and L-glutamate (Foster, Fagg, Mena and Cotman, 1981). Sharif and Roberts (1981) using synaptic membranes prepared from rat cerebellum found that specific L-[^{3}H]aspartate could be readily displaced by unlabelled L-aspartate (D-isomer half as active) whereas compounds such as DL-quisqualate, DL-4-fluoroglutamate and 2APB which are good displacers of L-[^{3}H]-glutamate binding were only weakly active against the aspartate system. In this study NMDA was ineffective as a displacer although in another (Mitchell and Redburn, 1982) NMDA and 2APB were used respectively to differentiate between L-[^{3}H]-aspartate and L-[^{3}H]-glutamate binding sites.

Specific binding sites for DL-[^{3}H]kainate have also been identified in brain synaptic membranes (Simon, Contrera and Kuhar, 1976). DL-[^{3}H]Kainic acid binding was most potently displaced by DL-quisqualate (as well as cold DL-kainate:London and Coyle, 1979). L-glutamate was next most potent being 25 times (Simon, Contrera and Kuhar, 1976) or 40 times (London and Coyle, 1979) less potent than DL-kainate. These latter authors also showed that D-glutamate was much less effective than L-glutamate, DL-dihydrokainate was much less effective than DL-kainate and that D- and L-aspartate,

glutamic acid diethylester (GDEE) and 2APB were ineffective as displacers.

GABA

Evidence of a rôle for GABA in central synaptic transmission is also provided by studies using CNS membrane fragments to locate and characterize neuronal GABA recognition sites. As with ligand binding studies employing labelled excitant amino acids, the use of tritiated GABA or the more potent naturally-occurring analogue, muscimol, in the presence or absence of relatively high concentrations of unlabelled GABA, has identified the existence of GABA binding sites in most regions of the CNS (Enna, 1978). Although not found in the rat (Enna and Snyder, 1975) there is a close correlation in the human and monkey CNS between the distribution of GABA binding sites and the activity of the GABA synthesizing enzyme, GAD (Enna, Kuhar and Snyder, 1975) and in the latter species Na^+-independent binding (which is thought to represent attachment to postsynaptic sites) parallels regional variations in endogenous GABA levels (Enna, Kuhar and Snyder, 1975).

Many studies using radiolabelled ligands have investigated binding in the presence of Na^+ (De Feudis, 1973, 1974; De Feudis and Black, 1973) and it has been suggested that this Na^+-dependent binding may represent interaction at a GABA transport site that perhaps involves glial cells (Enna and Snyder, 1975). Separation of Na^+-dependent from

Na^+-independent binding can be achieved by extensive washing of the membranes to remove Na^+, and freezing and thawing procedures which virtually inactivate Na^+-dependent but enhance Na^+-independent binding by two-fold (Young, Enna, Zukin and Snyder, 1976). Na^+-independent binding is also enhanced up to 5-fold by treatment of the membrane fragments with the detergent Triton X-100 (Enna and Snyder, 1977).

Microelectrophoretic studies (discussed later) have shown that the phthalide isoquinoline bicuculline was capable of antagonizing GABA-evoked inhibition of central neurones. Bicuculline appears to bind to the GABA recognition site (Mohler and Okada, 1978; Zukin, Young and Snyder, 1974; Bhattacharyya, Madyastha and Bhattacharyya, 1981) and has also been used to distinguish Na^+-independent from Na^+-dependent binding. Bicuculline has an ED_{50} of approximately 5 µM for inhibiting Na^+-independent GABA binding and completely abolishes binding at 100 µM. On the other hand Na^+-dependent GABA binding is 30 times less sensitive to bicuculline with an ED_{50} of 130 µM and maximal inhibition at >1 mM (Young, Enna, Zukin and Snyder, 1976).

Binding studies have also been useful in providing information into the structural requirements for GABA or its analogues to interact with either the postsynaptic recognition site or the GABA transport mechanism. For example, Krogsgaard-Larsen and Johnston (1978), investigated a series of compounds structurally related to muscimol on the Na^+ - independent binding of [^{3}H]GABA to membranes from rat brain.

Muscimol, homomuscimol and the bicyclic derivative 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP) which is an analogue of muscimol locked in a folded conformation, were relatively potent inhibitors of GABA binding as was the structurally related compound isoguvacine, a semi-rigid analogue of trans-4-aminocrotonic acid. Apart from muscimol, these analogues did not influence Na^+-dependent uptake of GABA in rat brain slices, whereas compounds such as guvacine and nipecotic acid inhibited uptake but did not influence GABA binding.

Whether or not the binding of tritiated GABA, muscimol or bicuculline represents interaction at a physiological GABA receptor, the ability of GABA-analogues to compete for Na^+-independent GABA binding to this recognition site correlates closely with their microelectrophoretic potency on central neurones. Thus, *in vitro* inhibitors of Na^+-independent GABA binding such as 3-aminopropane sulphonic acid (3APS), imidazole acetic acid and 3-hydroxy-GABA (Greenlee, Van Ness and Olsen, 1978) are also potent inhibitors of GABA sensitive neurones *in vivo* (Curtis and Watkins, 1960, 1961).

Microelectrophoretic Studies

Excitant amino acids

The original microelectrophoretic investigations (Curtis, Phillis and Watkins, 1959, 1960; Curtis and Watkins, 1960, 1961, 1963) demonstrated that certain acidic amino acids had excitatory effects on mammalian spinal neurones. In addition

to these reports further investigations showed essential structure-activity relationships (Crawford and Curtis, 1964; Krnjevic and Phillis, 1963) although stereospecificity did not appear to determine activity in most compounds since, for example, the stereoisomers of glutamate, aspartate and cysteate did not differ markedly in potency. However, exceptions to this observation were found to exist. Thus the D-isomers of homocysteate and the N-methyl analogue of aspartate are markedly more potent than the L-isomers (Curtis and Watkins, 1960, 1963).

It was initially considered that both L-glutamate and L-aspartate evoked excitation of central neurones through the same receptor, it being suggested (Van Gelder, 1971) that L-aspartate reacted with this receptor in an extended form and L-glutamate assumed a folded conformation in order to interact. Curtis and Watkins (1965) had noted that other dicarboxylates which had four carbon atom chains and which were otherwise identical to L-glutamate and L-aspartate displayed no excitant activity, the distance between terminal cationic and anionic groups being in excess of the equivalent distance in the folded glutamate or aspartate molecule. Because of the possible conformations that these endogenous dicarboxylates could adopt, studies were carried out using structurally rigid, or semi-rigid, analogues of glutamate in which the number of possible conformations was reduced. Some of the first compounds used were in fact naturally occurring conformationally-restricted analogues of glutamate. These substances included DL-kainate, DL-quisqualate and DL-ibotenate

and were shown to powerfully excite central neurones (Johnston, Curtis, de Groat and Duggan, 1968; Shinozaki and Konishi, 1970). This work provided further information on structure-activity relationships and coupled to the development of amino acid antagonists and the observations on the differential sensitivity of feline spinal interneurones to L-aspartate and L-glutamate, first provided microelectrophoretic evidence for the existence of separate populations of receptors for these amino acids. Thus feline Renshaw cells were more sensitive to electrophoretically-administered L-aspartate than to L-glutamate, whereas spinal dorsal horn interneurones were more sensitive to L-glutamate than to L-aspartate (Duggan, 1974). It was pointed out by Johnston, Curtis, Davies and McCulloch (1974) that kainate may be a more specific agonist for a glutamate receptor than glutamate itself which could adopt a multiplicity of conformations and thus possibly interact with more than one type of receptor. In particular Johnston, Curtis, Davies and McCulloch (1974) suggested that kainate may not be able to adopt a conformation suitable for interaction with the same receptors as those activated by NMDA and that NMDA was too small a molecule to interact with the glutamate receptor. With this in mind McCulloch, Johnston, Game and Curtis (1974) found that spinal interneurones were relatively more sensitive to DL-kainate than Renshaw cells which were more sensitive to NMDA.

Early studies with amino acid antagonists (Haldeman and McLennan, 1972; Haldeman, Huffman, Marshall and McLennan, 1972) suggested that GDEE might prove to be useful in deter-

mining the presence of receptors for glutamate. Subsequent studies however have expressed reservation on the selectivity of GDEE primarily due to the concurrent antagonism of responses to DL-homocysteate and L-aspartate (Curtis Duggan, Felix, Johnston, Tebecis and Watkins. 1972) and non-amino acid excitation by acetylcholine (ACh: Clarke and Straughan, 1977). Similarly, caution has been expressed regarding the claims that 1-hydroxy-3-aminopyrolid-2-one(HA-966, Davies and Watkins, 1973) and L-5,6-dimethoxyaporphine (nuciferine: Duggan, Lodge, Biscoe and Headley, 1973) were as selective for L-glutamate as claimed (McLennan and Wheal. 1976). The possibility that nuciferine, which did not differentiate between the excitatory effects of L-glutamate and ACh on Renshaw cells in the cat (Duggan, Lodge, Biscoe and Headley, 1973) selectively depressed the excitation of feline Renshaw cells by ACh and DL-kainate more than that by NMDA (Davies and Polc, 1979) has also been challenged (Curtis, Lodge and Bornstein, 1979).

Investigations with both organic and inorganic compounds eventually provided a more selective antagonism of L-aspartate excitation (which was more selective for NMDA). Thus divalent metal ions (Ault, Evans, Francis, Oakes and Watkins, 1980 Evans, Francis and Watkins, 1977; Davies and Watkins, 1977) such as Co^{2+}, Mn^{2+} and Mg^{2+} depressed responses in the frog and cat spinal cords to NMDA while having little or no effect on responses to DL-kainate or DL-quisqualate. Responses to L-aspartate were somewhat more sensitive to Mg^{2+} than were responses to L-glutamate though the major

part of the response to each of these putative transmitters was resistant to Mg^{2+}.

It had been shown that the glutamate homologue DL-α-aminoadipate was considerably weaker as an excitant of feline spinal neurones than L-glutamate when administered by microelectrophoresis, and since the D-enantiomorphs of glutamate and aspartate are as, or somewhat less, active as neuronal excitants than the naturally-occurring L-isomers (Curtis and Watkins, 1960) it was suggested that D-α-aminoadipate (DαAA) might possess antagonistic activity (Hall, McLennan and Wheal, 1977). Thus while L-monoaminodicarboxylates of chain length up to 8 carbon atoms (L-α-aminosuberate) have all been found to be agonists (Evans, Francis, Hunt, Oakes and Watkins, 1979), the D-isomers such as D-α-AA, D-α-aminopimelate (DαAP) and D-α-aminosuberate (DαAS) and the diaminodicarboxylate, α,ε-diaminopimelate demonstrated selective antagonism of NMDA-(and to a lesser extent L-aspartate-) evoked excitation of neurones in the cat spinal cord (Biscoe, Evans, Francis, Martin, Watkins, Davies and Dray, 1977; Biscoe, Davies, Dray, Evans, Martin and Watkins, 1978) rat thalamus (McLennan and Hall, 1978) and the isolated spinal cord of the frog (Evans, Francis, Hunt, Oakes and Watkins, 1979).

Although these antagonists showed minimal separation of responses to L-glutamate and L-aspartate, the existence of at least two different populations of amino acid receptor was proposed because of the minimal depression by these compounds of excitation by either DL-quisqualate or DL-kainate at concentrations of antagonist that markedly reduced excitation

by NMDA. A similar conclusion was reached in other studies (Hicks, Hall and McLennan, 1978; McLennan and Lodge, 1979; Davies and Watkins, 1979; Evans, Francis, Hunt, Oakes and Watkins, 1979) that demonstrated a converse ranking of amino acid agonists in order according to their susceptibility to blockade by GDEE or DαAA.

However, the existence of a third receptor for kainate also has support on the grounds of its minimal sensitivity to both GDEE and DαAA (Hicks, Hall and McLennan, 1978) and the conduction mechanisms underlying depolarization by this compound (Lambert, Flatman and Engberg, 1981; MacDonald and Wojtowicz, 1980: see Section c). The lack of a specific antagonist for this compound has hampered progress in this area but investigations carried out in this study with a series of new amino acid antagonists will add support to the proposal of a third receptor for kainate.

More systematic design studies have concentrated on structural modifications to the ibotenate molecule (a compound which paradoxically is a glutamate analogue structurally, but antagonized by DαAA and only minimally by GDEE: McLennan and Lodge, 1979) in order to produce more selective agonists for the central glutamate and aspartate receptors (Krogsgaard-Larsen, Honoré, Hansen, Curtis and Lodge, 1980; 1981). These investigations produced among others, the potent GDEE-sensitive compounds DL-4-bromo-homo-ibotenate and DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA).

In this thesis a study has been carried out with the stereoisomers of AMPA as well as the racemic mixture of some recently synthesized and related compounds in an attempt to gain additional information into the stereochemical and conformational requirements of the central amino acid receptors. As well as this, GDEE and the new NMDA antagonist 2-amino-5-phosphonovalerate (2APV: see Chapter 4) were employed to determine the class of receptor activated by these compounds.

GABA

The neutral amino acid GABA and many of its analogues have now been shown microelectrophoretically to inhibit the firing of several different classes of neurones in the vertebrate CNS. For example, GABA has inhibitory actions on cat motoneurones, dorsal horn interneurones and Renshaw cells (Curtis, Phillis and Watkins, 1959; Curtis and Watkins, 1960, 1961), cat and rat cerebral cortical neurones (Krnjević and Phillis, 1963; Crawford and Curtis, 1964; Biscoe, Duggan and Lodge, 1972) and cat cerebellar Purkinje neurones (Kawamura and Provini, 1970, Curtis, Duggan, Felix, Johnston and McLennan, 1971) and cuneate neurones (Kelly and Renaud, 1971). As had been shown for the action of acidic amino acids specific structural features were required for depressant action (Curtis and Watkins, 1960). Optimum separation of the basic and acidic groups was two or three carbon atoms. The acidic function could be sulphonic or carboxylic in the case

of the two carbon connecting chain and activity was abolished if the acidic or basic groups were not free and reduced or abolished by substitution within the intermediate carbon chain or upon the amino group.

Unlike the excitant amino acids structural manipulation of the GABA molecule has been unsuccessful in producing microelectrophoretic antagonists of this compound. However, antagonism of the postsynaptic effect of GABA has been demonstrated with the naturally-occurring compounds picrotoxin and bicuculline. Picrotoxin, originally isolated from the moonseed family of plants, showed inconsistency in its effects on GABA inhibitions when ejected microelectrophoretically, a result which may be due to difficulties encountered in passing sufficient picrotoxin from the micropipette (Curtis and Johnston, 1974). Although picrotoxin produces occasional and selective antagonism of GABA responses in the spinal cord (Engberg and Thaller, 1970) and on presynaptic terminals in the cuneate nucleus (Galindo, 1969) picrotoxin did not affect the depressant action of GABA on cat medullary reticular formation (Hösli and Tebēcis, 1970), spinal (Curtis, Hösli and Johnston, 1968; Curtis, Duggan and Johnston, 1969) or cortical neurones (Krnjević, Randić and Straughan, 1966). It has also been claimed that electrophoretically-administered picrotoxin consistently blocked the inhibitory action of glycine on 95% of spinal neurones on which it was tested (Davidoff and Aprison, 1969).

Bicuculline, a convulsant (Meldrum and Horton, 1971), phthalide isoquinoline alkaloid isolated from Corydalis

species was first reported by Curtis, Duggan, Felix and Johnston (1970) to antagonize the inhibitory effects of microelectrophoretic GABA when ejected from micropipettes onto single neurones in the cat spinal cord and cerebral and cerebellar cortices. The inhibitory effects of glycine, L-α-alanine, taurine and noradarenaline were not affected by electrophoretically-administered bicuculline whereas this compound reversibly antagonized responses to GABA and some of its analogues (Curtis, Duggan, Felix and Johnston, 1971). Bicuculline also antagonized the action of microelectrophoretic GABA on neurones in the lateral vestibular nucleus, the ventrobasal thalamus and the hippocampal cortex (Curtis, Duggan, Felix, Johnston and McLennan, 1971).

Evidence that this inhibition of cell firing by GABA involves a hyperpolarization associated with a change in Cl^- conductance will be discussed in Section c. However GABA has also been implicated as the transmitter released at axoaxonic synapses (see Section a) on afferent endings in the mammalian CNS (Eccles, Schmidt and Willis, 1963a) where it appears to have a depolarizing action. Such an effect is manifested as an increase in excitability of the terminal portion of the afferent fibre (Gmelin and Cerletti, 1976; Sverdlov and Kozhechkin, 1975) and the involvement of GABA in primary afferent depolarization and the relationship with presynaptic inhibition will also be discussed in Section c.

Although different effects on membrane potential are seen in central neurones and afferent endings in response to GABA, the receptor on both structures appears pharmacologically

similar with regards to antagonism by bicuculline (Curtis Bornstein and Lodge, 1980).

As well as its central effects GABA depolarizes mammalian sympathetic ganglia (de Groat, 1970; Bowery and Brown, 1974 Adams and Brown, 1975) sensory neurones (Gallagher, Higashi and Nishi, 1978; de Groat, Lalley and Saum, 1972; de Groat, 1972) and unmyelinated axons in peripheral nerve trunks (Brown and Marsh, 1978). However, despite the fact that some of these effects are antagonized by bicuculline and picrotoxin (de Groat, 1972; Gallagher, Higashi and Nishi, 1978; Brown and Marsh, 1978) the receptors on these structures may be pharmacologically different from those activated on either central neurones or at axoaxonic synapses on afferent terminals. Published results of the relative effects in vitro of a series of GABA analogues on rat superior cervical ganglion neurones (Bowery, Collins, Hudson and Neal, 1978; Bowery and Brown, 1974, Bowery and Jones, 1976) appear different from their relative potencies as inhibitors of mammalian spinal neurones (Curtis and Watkins, 1960, 1961; Johnston, Curtis, Beart, Game, McCulloch and Twitchin, 1975; Krogsgaard-Larsen, Falch, Schousboe, Curtis and Lodge, 1980; Krogsgaard-Larsen, Johnston, Lodge and Curtis, 1977) and a recent investigation (Curtis, Bornstein and Lodge, 1980) in which these differences were analysed also suggested dissimilarities between GABA receptors on central neurones and afferent terminals on the one hand and those responsible for GABA's depolarizing action on feline dorsal root ganglion cells in vitro.

Thus subclasses of bicuculline-sensitive GABA receptors may be present in mammalian nervous tissue and the existence of bicuculline-insensitive GABA receptors on peripheral autonomic nerve terminals (Bowery, Doble, Hill, Hudson, Shaw, Turnbull and Warrington, 1981) and synaptic membranes (Bowery, Hill and Hudson, 1981) has also been reported.

Rôle of amino acids in synaptic transmission

Excitant amino acids

Hand in hand with these studies on the antagonism of microelectrophoretically-evoked excitation by acidic amino acids has been experiments designed to determine the involvement of particular amino acids as transmitters in central nervous pathways. Undoubtedly investigations in this area have been hampered by the lack of a specific glutamate antagonist. Irrespective of this, however, antagonism of synaptic excitation has been demonstrated in the mammalian (Biscoe, Davies, Dray, Evans, Martin and Watkins, 1978; Lodge, Headley and Curtis, 1978; Davies and Watkins, 1979) and amphibian spinal cord (Evans, Francis, Hunt, Oakes and Watkins, 1979) the cochlear nucleus (Martin, 1980), the caudate nucleus (Stone, 1979) the hippocampus (Hicks and McLennan, 1979) and in the cerebellum (Stone, 1979).

Of particular importance to this present investigation are results obtained in the cat spinal cord showing that DαAA and DαAS (Davies and Watkins, 1979) produced partial blockade of the late (presumed on its central latency to be poly-

synaptic) excitation of cat spinal interneurones. Based on both this and neurochemical evidence L-aspartate has been proposed as the transmitter released by some spinal excitatory interneurones, and L-glutamate by some primary afferents, a proposal that is supported by observations on the greater sensitivity of Renshaw cells to L-aspartate, cells known not to receive a monosynaptic innervation from primary afferent fibres in the cat (Duggan, 1974). Further evidence for different transmitters has been provided by recent evidence of the selective, and presumed presynaptic, reduction of the monosynaptic excitation of spinal interneurones by the GABA analogue (-)-β-p-chlorophenyl GABA (Curtis, Lodge, Bornstein and Peet, 1981).

Because of the uncertainties over the lack of specificity and relatively low potency of proposed glutamate antagonists and in particular GDEE (Davies and Watkins, 1979) which affects the amplitude of the action potential at higher concentrations (Hicks, Hall and McLennan, 1978), there has been less conclusive evidence for the involvement of glutamate in synaptic excitation. However, GDEE depresses synaptic excitation in several central regions including the cerebral cortex (Stone, 1973), thalamus (Haldeman, Huffman, Marshall and McLennan, 1972) and hippocampus (Wheal and Miller, 1980).

Blockade of the synaptic event evoked by stimulation of a particular pathway by compounds with known microelectrophoretic antagonistic properties provides important information on the characteristics of the receptor that might be activated by a released transmitter but does <u>not</u> provide information on the

nature of that substance. Nevertheless, compounds have been synthesized recently that have been demonstrated microelectrophoretically to have highly selective patterns of antagonism and the effect of these substances on the synaptic excitation of interneurones and Renshaw cells will be investigated in this study.

GABA

A rôle for GABA in postsynaptic inhibitory actions in many regions of the mammalian brain has been documented (see Curtis, 1978b and review by Curtis and Johnston, 1974). For example, cortical pyramidal cells can be inhibited by microelectrophoretic GABA (Crawford and Curtis, 1964) and both this and the inhibition of these neurones following electrical stimulation of the pyramid or the surface of the cerebral cortex or by chemical stimulation of intercortical stellate cells can be reduced by microelectrophoretic bicuculline (Curtis and Felix, 1971).

GAD activity in the cerebellar cortex (Albers and Brady, 1959) and the uptake of [^{3}H]GABA after direct injection into the cerebellum is largely associated with intrinsic interneurones (Fonnum, 1972) that have an inhibitory function on the firing of Purkinje cells (Eccles, Ito and Szentagothai, 1967). Since bicuculline is capable of antagonizing both the inhibition in response to microelectrophoretic GABA and that evoked synaptically by electrical stimulation of the surface of the cerebellar cortex (Curtis and Felix, 1971) compelling

evidence is provided for the involvement of GABA in the inhibition of Purkinje cells.

Use of the antagonists bicuculline, picrotoxin and strychnine has been important in determining the likely transmitters of synaptic inhibition in the cat spinal cord. Thus, the recurrent or "mutual inhibition" of Renshaw cells (Curtis, Game, Lodge and McCulloch, 1976), the Renshaw cell inhibition of Ia interneurones (Belcher, Davies and Ryall 1976) mediating strychnine-sensitive short latency inhibition of spinal motoneurones (Hultborn, Jankowska and Lindström, 1971) and the short-latency inhibition of ventral group Ia-excited spinal interneurones by volleys in afferent fibres (Lodge Curtis and Brand, 1977) are all sensitive to strychnine and therefore, presumably are mediated by glycine. However, the longer latency and duration inhibition of Ia-excited spinal interneurones and motoneurones is reduced by bicuculline (Lodge, Curtis and Brand, 1977 Curtis, Duggan, Felix and Johnston, 1971) and would appear to involve GABA.

The longer latency and duration inhibition of motoneurones can be measured as a reduction in amplitude of either intracellularly recorded epsps or monosynaptic reflexes (Eccles, Schmidt and Willis, 1963c) and evidence considered in Section a on the existence of GAD-containing structures axodendritically, axosomatically and axoaxonally to motoneurones in the mammalian spinal cord, provides morphological (Conradi, 1969a,b) and immunocytochemical support (McLaughlin, Barber, Saito, Roberts and Wu, 1975) for a pre- and/or postsynaptic mechanism underlying this inhibition.

(c) Interaction with postsynaptic receptors and the underlying ionic mechanism(s) of action

Excitant amino acids

Early studies (Curtis, Phillis and Watkins, 1959, 1960, 1961; Curtis and Watkins, 1960, 1963) using intracellular recordings from central neurones suggested that an increase in neuronal membrane permeability was responsible for the depolarization in response to microelectrophoretic L-glutamate, L-aspartate and DL-cysteate, and if depolarization was greater than the threshold for cell firing the amino acid evoked action potentials. However, it was observed during these investigations that some cells displayed phenomena that appeared related to intense firing in response to higher currents of excitant amino acids. These phenomena included a reduction of the firing frequency towards the end of ejection of a compound despite a constant flow of electrophoretic current (see also Curtis and Ryall, 1966b). Also, in cells with a background firing rate (in the absence of pronounced changes in the shape of the action potential) excitation by amino acids (Curtis, Phillis and Watkins, 1960) and acetylcholine (Curtis and Ryall, 1966b) was often followed by a period of reduced background excitability lasting for many seconds after termination of the excitant current. Reductions in the size of the action potential were also observed, and if excitation was sufficiently intense, cessation of firing could occur. This so-called "depolarization block" (Curtis, Phillis and Watkins, 1960) was attributed to inactivation of the sodium carrier system so

rendering portions of the membrane inexcitable. Since the results of a recent study (McLennan and Liu, 1981) have proposed a different cause for the reduced excitability following termination of the excitant current, a further study was made in this thesis on the mechanism responsible for these observations.

Although these early studies suggested that at least an increase in the neuronal membrane permeability to Na^+ (P_{Na}) was responsible for the depolarizing actions of excitant amino acids, later investigations, using similar techniques, showed that L-glutamate depolarization was associated at low doses with either increases in membrane conductance (Gm: feline red nucleus neurones: Steinberg, Altmann and ten Bruggencate, 1974) or no detectable change (feline motoneurones and interneurones: Bernardi, Zieglgänsberger, Herz and Puil, 1972) and at higher doses with only increases in Gm (Bernardi, Zieglgänsberger, Herz and Puil, 1972; Zieglgänsberger and Puil, 1973). These results have been confirmed in more recent studies on hippocampal pyramidal cells (Hablitz and Langmoen, 1982) which also demonstrated that when external Na^+ concentration was reduced the size of the L-glutamate response decreased, a result that supports previous investigations showing the Na^+-dependence of amino acid-induced depolarizations (Barker and Nicoll, 1973; Evans, Francis and Watkins, 1977).

An initial study (Engberg, Flatman and Lambert, 1975) demonstrated, however, that there were differences in the depolarizing mechanisms of L-glutamate and DL-homocysteate,

the depolarization by this latter compound being accompanied by a marked decrease in Gm. On the basis of this and other studies (Engberg, Flatman and Lambert, 1978, 1979a,b; Lambert, Flatman and Engberg, 1978, 1981) excitant amino acids were allocated by these authors to one of 3 categories depending upon the nature of their effects upon cat spinal neurones.

Group I contained L-glutamate, L-aspartate, DL-quisqualate and L-homocysteate. Depolarization by this group showed fast onset and recovery with minimal change in absolute Gm. However depolarizing the membrane by passing tonic depolarizing current through the recording electrode to control for the influence of rectification, revealed a small increase in Gm. Biphasic changes in Gm were seen with large, long lasting applications of excitant. These consisted of an early decrease, followed by a slowly increasing Gm as the depolarization was maintained. It was suggested (Engberg, Flatman and Lambert, 1979a) that the high conductance phase may be a manifestation of low affinity amino acid uptake. These excitants usually produced only short periods of repetitive firing.

The second group consisted of the amino acids NMDA, DL-ibotenate and D-homocysteate. The depolarizations evoked by these compounds were slow in onset and recovery and were accompanied by a large, stable decrease in Gm. When depolarization was sufficient to reach the threshold for spike generation, stable repetitive firing resulted, which was capable of lasting throughout agonist applications of many minutes.

The responses of the third group which consisted of the potent agonist DL-kainate bore no resemblance to those observed in the other two categories. Responses to DL-kainate were characterized by a slow onset and very slow recovery which was usually incomplete unless very small ejecting currents were used. The rising phase of DL-kainate depolarizations was usually, but not always, accompanied by a short period of repetitive firing, while Gm increased. During continued DL-kainate ejection the membrane potential plateaued. The neurone was then completely inexcitable and Gm immeasurably high.

Investigations by intracellular recordings from single neurones of foetal mouse spinal cord or brain grown dissociated in tissue culture (MacDonald and Wojkowicz, 1980; 1982) suggested that amino acids could be allocated to one of two groups depending upon the conductance change they evoked. The first group, including, L-glutamate, L-aspartate, D-homocysteate, NMDA and DL-ibotenate, produced a primary effect that was depolarization accomplished by an apparent decrease of Gm although in most instances an expected increase in Gm was also recorded, especially if membrane potential was reduced by tonic depolarization. DL-Kainate, however, never decreased Gm and invariably caused an increase, an effect also observed with DL-quisqualate (MacDonald and Porietis, 1982). Another difference in the kainate Gm and that evoked by other amino acids was that both changes in conductance in response to these latter compounds could be blocked by the receptor antagonist α-aminoadipate whereas the conductance change induced by DL-kainate was unaffected.

The decrease in Gm upon current depolarization with for example NMDA or D-homocysteate might be explained by development of a persistent inward calcium current which could oppose the normal outward current (Lux and Schubert, 1975). However the use of Ca^{2+} antagonists in the experiments of Engberg, Flatman and Lambert (1979b) did not support this suggestion. These latter authors also demonstrated (Engberg, Flatman and Lambert, 1979a) that DL-homocysteate responses (which showed a decreased Gm and therefore probably reflected the response to D-homocysteate) were insensitive to changes in the internal Cl^- concentration but were reduced by increasing the external K^+ concentration. Thus, the decrease in Gm seen with the group two amino acids (for example, D-homocysteate) was probably mediated by a decrease in K^+ conductance and that the availability of this conductance channel was potential dependent.

In addition to criticism of the rôle of amino acids as central transmitters on biochemical grounds, further objections, relating particularly to L-glutamate, have centred around the apparent discrepancy between the reversal potential of epsps and amino acid-evoked potentials. The early studies of Coombs, Eccles and Fatt (1955) and the more recent investigations of Engberg and Marshall (1979) demonstrated that the Ia epsp (which is thought on neurochemical grounds to be mediated by L-glutamate or a similar transmitter) reversal potential is approximately 0 mV while the reversal potential for such amino acid-induced depolarization is considerably more negative (less depolarized; Curtis, 1965; Zieglgänsberger

and Puil 1973) However it has been realised that a high proportion of excitatory synapses are probably located dendritically and thus the receptors activated by synaptically-released transmitter. with subsequent transient alteration in the membrane potential. may represent a different population (one which might be inaccessible) to those activated by electrophoretically-applied amino acid in the vicinity of the electrode impaling the soma. Thus spurious differences in the estimated reversal potential for epsps and amino acid-induced depolarizations might be recorded (Curtis and Johnston, 1974, Zieglgansberger and Puil, 1973).

In more recent investigations, however, a closer correlation has been reported between the reversal potential for the epsp and that for L-glutamate depolarization in the hippocampal slice preparation. Orthodromic stimulation of stratum radiatum elicited a mixed epsp-ipsp. When the ipsp is reduced or inhibited the pure excitatory synaptic event in CA3 pyramidal neurons has a reversal potential at or near 0 mV (Johnston, Brown, Hablitz and Lebeda, 1980) which is very similar to the value of -1.5 ± 6.5 mV for the reversal potential of L-glutamate depolarization in the same preparation (Langmoen and Hablitz, 1981; Hablitz and Langmoen, 1982).

GABA

The inhibitory effect of GABA on the firing of central neurones (outlined in Section b) seems primarily to be an action on the postsynaptic membrane since spontaneous,

antidromic, synaptic- and chemically-induced firing of spinal α and γ motoneurones, dorsal and ventral horn interneurones and Renshaw cells is reduced by microelectrophoretic GABA (Curtis, Phillis and Watkins, 1959). The synaptic- or GABA-evoked hyperpolarization of motoneurones (Curtis, Hösli, Johnston and Johnston, 1968) and interneurones (Bruggencate and Engberg, 1968) by GABA recorded by intracellular electrodes, appears to involve an increase in membrane conductance to Cl^- and possibly K^+ (Curtis, Hösli, Johnston and Johnston, 1968, Bruggencate and Engberg, 1968).

In addition to this hyperpolarizing action at central neurones GABA has a depolarizing effect on the terminals of afferent fibres in the spinal cord (Gmelin and Cerletti, 1976; Sverdlov and Kozhechkin, 1975) and cuneate nuclei (Davidson and Southwick, 1971) and peripherally at various sympathetic (De Groat, 1970; Adams and Brown, 1975) and sensory (De Groat, 1972) ganglia. The former of these effects in the spinal cord has led to the implication of GABA (Eccles, Schmidt and Willis, 1963a) in the production of the externally-recorded dorsal cord potentials, dorsal root potentials and dorsal root reflexes, phenomena associated with the depolarization of the terminals of primary afferents in response to repetitive volleys in segmental afferents.

Previous experiments (Gasser and Graham, 1933; Hughes and Gasser, 1934) had shown that following an afferent volley to the spinal cord, a prolonged positive wave was recorded from the dorsal surface of the cord and had the same time course as the inhibition of flexor reflexes (Eccles and Sherrington,

1931). Such an afferent volley also elicited a depolarization of the intramedullary portion of adjacent primary afferents which could be recorded as an electrotonic potential (dorsal root potential) or if sufficiently intense, as a dorsal root reflex (Barron and Matthews, 1938).

The possibility that this mechanism was presynaptic and different from the shorter latency direct inhibition of 1a motoneurones (Eccles, Fatt and Lundgren, 1956) was suggested by experiments (Frank and Fuortes, 1957) in which monosynaptic epsps recorded from gastrocnemius motoneurones in response to a single stimulus to the homonymous nerve were reduced by up to 50% it this test stimulus was preceded (approximately seven msec) by a volley in hamstring afferents. This reduction of the epsp occurred without changes to the excitability of the gastrocnemius motoneurones. These results were confirmed by Eccles, Eccles and Magni (1961) who established that the reduction of the epsp had the same time course as the dorsal root potentials and that the falling phase of the monosynaptic epsp was unaltered during the inhibition.

The development of a technique (Wall, 1958) which allowed measurement of the changes in excitability of afferent terminals and fibres by directly stimulating such structures in the spinal cord with a microelectrode and recording the size of the antidromically-conducted volley has been useful in identifying the source and type of fibre receiving PAD . Changes in the excitability of terminals would thus determine the number that would fire in response to a given pulse through the microelectrode and this number would be reflected

in the size of the antidromic impulse. Using this technique it has been found that afferent fibres are maximally depolarized in the region where these fibres are thought to terminate, with, for example, maximal depolarization of cutaneous afferents occurring in the more superficial laminae of the dorsal horn (Wall, 1958, 1962). Important for the studies carried out in this present investigation are the observations that extensor group Ia afferents are maximally depolarized by volleys in nerves from flexor muscles in the motoneuronal nucleus (Eccles, Magni and Willis, 1962; Eccles, Schmidt and Willis, 1963b) a region where these afferents are known to terminate (Brown and Fyffe, 1978).

PAD has also been demonstrated by directly inserting a fine microelectrode into single fibres in the cat spinal cord (Koketsu, 1956; Eccles and Krnjević, 1959). These recordings, while allowing identification of the type of afferents being depolarized, do not provide information on the site of the original depolarization since the point of penetration of the electrode is undoubtedly in the larger unbranched portion of the fibre.

The depolarization of afferent terminals is suggested to presynaptically reduce the amount of transmitter substance released in response to a given impulse. Since the amount of transmitter reduced per impulse from terminals is influenced by the presynaptic membrane potential the degree of depolarization of the terminal might be expected to be an important factor governing release (Hubbard, 1970). Changes in the amplitude of the action potential invading the terminal region may be

important, as well as more direct effects on the release mechanism and the amount of transmitter available for release. In the squid stellate ganglion a 10-30 mV change in the presynaptic potential produces an approximately ten-fold change in the amplitude of the postsynaptic potential, a 30% reduction of the former almost abolishing transmission (Katz and Miledi, 1971; Takeuchi and Takeuchi, 1962).

The possibility also exists that this longer latency and duration (200-300 ms) inhibition may be postsynaptic in nature. This "remote inhibition" (Frank, 1959) may be occurring at synapses on dendrites so distant from the soma of the motoneurone that changes in membrane potential and conductance are not readily detected by an intracellular electrode in the soma (Granit, 1968; Kellerth, 1968). In fact both a pre- and postsynaptic mechanism has been proposed (Cook and Cangiano, 1970, 1972). In cats anaesthetized with pentobarbitone, recordings were made from gastrocnemius, flexor digitorum longus and flexor hallucis longus motoneurones in response to stimulation of the homonymous muscle nerve.

Maximal reduction of the evoked epsp was achieved at group Ia stimulus strength stimulation (4 volleys) of the posterior biceps and semitendinosus nerves (PBST). Group I PBST conditioning volleys also evoked small, long-lasting, hyperpolarization of the motoneurones. This ipsp, which was suggested to be of dendritic origin continued to increase in amplitude with group II and III strength conditioning volleys. Thus although maximum epsp depression occurred at group I stimulus strength there was no effect on repetitive discharge,

whereas the evoked postsynaptic inhibitory effects and the inhibition of repetitive discharge of the motoneurone increased in parallel.

The longer latency of this type of inhibition suggests that a polysynaptic relay of at least two interneurones is involved (Curtis, 1978a). PAD can be evoked in Group Ia afferents by intraspinal stimuli in laminae V and VI, a response possibly augmented by stimulation of last order interneurones in this region (Jankowska, McCrea, Rudomin and Sykova, 1981). The final interneurone on this pathway may be gabergic, the immunocytochemical location of GAD at axoaxonic, axosomatic and axodendritic synapses (McLaughlin, Barber, Saito, Roberts and Wu, 1975) on motoneurones supporting this proposal. Pharmacological investigations on the rôle of GABA in PAD have shown that it is capable of depolarizing terminals in the cat spinal cord whether applied topically (Eccles, Schmidt and Willis, 1963a) or electrophoretically (Gmelin and Cerletti, 1976; Sverdlov and Kozhechkin, 1975) and that this depolarization and the synaptically-evoked event are both reduced by picrotoxin (Eccles, Schmidt and Willis, 1963a) and bicuculline (Curtis, Duggan, Felix and Johnston, 1971; Levy and Anderson, 1972). The longer latency inhibitory hyperpolarization of motoneurones described by Kellerth (1968) was also reduced by picrotoxin but not by strychnine (see also Cook and Cangiano, 1972) and the administration of semicarbazide to acute spinal cats to reduce GABA levels suppresses dorsal root potentials and dorsal root reflexes (Bell and Anderson, 1972).

The mechanism of GABA's depolarization of peripheral structures, like its hyperpolarizing effect at central neurones, appears to involve a change in the membrane conductance to Cl^-. Thus, the depolarization of cultured rat superior cervical ganglia by GABA was accompanied by an increased membrane conductance (Obata, 1974) and a subsequent detailed analysis, in which intracellular recording from superior cervical ganglia in vitro was combined with alteration of the extracellular concentration of Cl^- indicated that GABA increased the membrane permeability to Cl^- which was apparently at equilibrium at a potential less depolarized than that of the resting membrane. Similar effects have been suggested to account for the depolarization by GABA of ganglion cells and presynaptic fibres in the bullfrog paravertebral sympathetic ganglia in vitro (Koketsu, Shoji and Yamamoto, 1974) effects that can be blocked by picrotoxin.

The effect of GABA on sensory ganglia, which are generally considered not to contain synapses appears to involve a similar mechanism to that seen with autonomic neurones. Depolarization of the lumbar dorsal root ganglia by GABA was reduced by bicuculline and picrotoxin but not strychnine (de Groat, 1972). A similar effect of GABA in bullfrog dorsal root ganglia in vitro is accompanied by an increased membrane conductance, alterations of the extracellular ion concentration suggesting that GABA increases Cl^- permeability (Nishi, Minota and Karczmar, 1974).

These results suggest, therefore, that for GABA to produce depolarization peripherally (and presumably in the

central terminations of primary afferent neurones) and hyperpolarization centrally, the intracellular concentrations of Cl^- in ganglia (and terminations) must be maintained at a higher level than that found in central neurones. An inwardly directed Cl^- pump maintaining intracellular levels greater than those expected from an equilibrium distribution has been proposed (de Groat, 1972; Nishi, Minota and Karczmar, 1974; Adams and Brown, 1975). In the isolated frog spinal cord piretanide, a drug which blocks active Cl^- transport in other systems, abolished the dorsal root depolarizing response to GABA and produced a small shift in the GABA equilibrium potential towards the resting membrane potential in dorsal root ganglion cells, a result which is consistent with a reduced inward pumping of Cl^- (Wojtowicz and Nicoll, 1982).

(d) Termination of the action of amino acids

Excitant amino acids

For the action of a transmitter or putative transmitter to be terminated, whether it be synaptically released or ejected electrophoretically, it must either be inactivated or removed from regions of contact with postsynaptic receptors. This might involve one of several processes including simple diffusion, extracellular enzymic modification, or the binding to extraneuronal sites with subsequent uptake into surrounding cellular elements. Any form of interference or blockade of these processes might be expected to increase the duration of

action of a synaptically-released transmitter and would presumably prolong or enhance the postsynaptic effect of this compound. If such an effect could also be achieved for an exogenously administered compound then this would add support to the rôle of this substance in transmission at that location.

Presumably because of what was known about the enzymic termination of cholinergic transmission, it might have been expected that specific enzymes would be located near excitatory synapses associated with the termination of excitation by excitatory amino acid transmitters. However, when L-glutamate, L-aspartate or L-cysteate were administered electrophoretically to the same interneurone (Curtis and Watkins, 1960), the intervals preceding the cessation of cell responses after the termination of the electrophoretic currents were virtually identical in each case, as, and perhaps more importantly, were the intervals for both (D) and (L) forms of glutamate and aspartate ions. This suggested that extracellular enzymic degradation of the applied acids was probably not a significant factor in determining the duration of action of these amino acids (Curtis, Phillis and Watkins, 1960). It is now realised that active uptake processes exist (Balcar and Jonston, 1972b; Benjamin and Quastel, 1976; Logan and Snyder, 1972, Charles and Chang, 1981; Yamamoto and Sawada, 1982) for the removal of amino acids from the extracellular space and that these mechanisms probably contribute to the inactivation of synaptically-released transmitters (Balcar and Johnston, 1972b).

The uptake of labelled L-glutamate and L-aspartate has also been used for identifying alleged glutamergic and aspartergic nerve endings (Divac, Fonnum and Storm-Mathisen, 1977; Iversen and Storm-Mathisen, 1976; Taxt, Storm-Mathisen, Fonnum and Iversen, 1977; Fonnum, Storm-Mathisen and Divac, 1981; Bromburg, Penney, Stephenson and Young, 1981). Uptake of labelled D-aspartate has also been demonstrated by the autoradiographic retrograde labelling of rat olivocerebellar climbing fibre neurones after its injection into the cerebellar cortex or deep nuclei (Wikland, Toggenburger and Cuénod, 1982).

Two kinetically distinct systems have been described for the uptake of excitatory amino acids into brain tissues or synaptosomes, the concentration of transmitter possibly determining which system would participate in the inactivation (Balcar and Johnston, 1972a). The low affinity system (characterized by a high Vmax:Km of 0.1-1.0 mM) is probably important in the inactivation of electrophoretically administered excitatory amino acids in the mammalian spinal cord (Cox, Headley and Watkins, 1977; Lodge, Curtis, Johnston and Bornstein, 1980). It is known that both glia and neurones possess both low and high affinity systems (Hertz, 1979) and that since high affinity transport systems (low capacity, Km of 1-50 μM) have not been detected for amino acids not thought to be transmitters (except proline), this mechanism appears to be specifically involved in the termination of synaptic transmission by these compounds (see Martin, 1976).

Early attempts at blocking uptake of excitant and inhibitory amino acids centred around the use of p-chloromercuriphenylsulphonate(PCMP). This compound showed no specificity, enhancing the inhibitory action of GABA, β-alanine and L- and D-α-alanine applied microelectrophoretically in the cat spinal cord (Curtis, Duggan and Johnston, 1970) and to inhibit the high affinity uptake of glycine and GABA into slices of cat spinal cord (Balcar and Johnston, 1973). At higher concentrations in the same studies this compound enhanced the excitant action of acidic amino acids and inhibited their uptake.

Studies by Balcar and Johnston (1972a) showed that uptake of L-glutamate into rat brain slices was not inhibited by either excitant amino acids which have an appreciably greater depolarizing action on central neurones than that of L-glutamate or antagonists of L-glutamate-induced excitation of central neurones. These and other studies (Roberts and Watkins, 1975) have therefore suggested that compounds inhibiting L-glutamate uptake by glia and nerve endings require different structural characteristics from those antagonizing the postsynaptic receptor effect of L-glutamate. Thus substances such as kainate and NMDA which are powerful neuronal excitants failed to inhibit L-glutamate uptake whereas weak excitants such as L-glutamic acid γ-hydroxamate and DL-aspartic acid β-hydroxamate were found at that time to be the strongest inhibitors of both glial and neuronal L-glutamate uptake (Roberts and Watkins 1975).

Studies designed to compare the time course of microelectrophoretically-applied glutamate analogues correlates well with information relating to their uptake. Thus, for, example D-homocysteate which is thought not to be a substrate for either high or low affinity uptake systems (Balcar and Johnston, 1972) has recovery times considerably longer than those amino acids such as L-glutamate, L-aspartate and D-aspartate which are accumulated by both high and low affinity systems (Cox, Headley and Watkins, 1977). The lack of, or relatively inefficient, uptake mechanisms for compounds such as NMDA, D-homocysteate, ibotenate, kainate (Curtis, 1979), 4-methylhomoibotenate (4MHI), α-amino-β-methyl-3-hydroxy-4-methyl-3-isoxazolepropionate (AMAA) and AMPA (Anis, Headley and Lodge, 1980) may be a contributing factor to the potency of these compounds.

Recently two compounds have been found to be useful as uptake inhibitors for excitant amino acids. The first, threo-3-hydroxy-L-aspartate has been found to be an extremely potent and selective inhibitor of the high affinity uptake of L-glutamate and L-aspartate in rat brain slices, its stereoisomer threo-3-hydroxy-D-aspartate being only marginally less effective (Balcar, Johnston and Twitchin, 1977). The stereoisomers of this compound have also been shown to potentiate the excitation of cat spinal neurones by electrophoretic L-glutamate and L-aspartate (Johnston, Lodge, Bornstein and Curtis, 1979).

DL-Dihydrokainate has also been shown to be useful as an uptake inhibitor, enhancing excitations by compounds taken up actively *in vitro* by CNS tissue (L-aspartate, D- and L-glutamate and L-homocysteate) and having no effect on those not taken up (acetylcholine, D-homocysteate, DL-kainate and NMDA; Lodge, Johnston, Curtis and Bornstein, 1979). Responses to the potent excitant DL-quisqualate are also enhanced by dihydrokainate and threo-3-hydroxyaspartate (Lodge, Curtis, Johnston and Bornstein, 1980) suggesting that DL-quisqualate may also be taken up into CNS tissue which would agree with the relatively fast recovery from its action.

GABA

The inactivation of the postsynaptic effect of GABA and the maintenance of low extracellular GABA levels is believed to take place via high affinity transport proceses in presynaptic nerve terminals (Bloom and Iversen, 1971; Iversen and Kelly, 1975; Storm-Mathisen, 1975) and glial cells (Henn and Hamberger, 1971, Iversen and Kelly, 1975; Schousboe, Hertz and Svenneby, 1977). Carrier mediated processes have been extensively studied *in vitro* using tissue slices, homogenates, subcellular fractions and cultured glial cells and neurones (see review by Martin, 1976) and has been found to be Na^+-dependent (Bennett, Logan and Snyder, 1972; Desarmenien, Feltz and Headley, 1980), temperature sensitive and can be inhibited

by metabolic poisons (Neal and Iversen, 1969; Iversen and Neal, 1968). The uptake of [^{3}H]GABA is primarily associated with nerve terminals (Iversen and Bloom, 1972) and the electrolytic destruction of a presumed GABA tract running from the globus pallidus to the substantia nigra results in a loss of uptake of radioactive GABA in the substantia nigra in association with a decrease in GABA levels (Hattori, McGeer, Fibiger and McGeer, 1973). Two structurally specific GABA transport systems have been identified, namely a low affinity (Km higher than 5 x 10^{-4}M) and a high affinity (Km lower than 5 x 10^{-5}M) with the latter process being Na^+-dependent (see Martin, 1976).

Different structural requirements appear to be necessary for binding to the receptor and transport sites (Schousboe, Thorbek, Hertz and Krogsgaard-Larsen, 1979) and GABA uptake can be separated into glial and neuronal components on the grounds of substrate specificity (Iversen and Kelly, 1975). In the former study the most selective inhibitors of [^{3}H]GABA receptor binding were (RS)-4,5-dihydromuscimol, muscimol, GABA, isoguvacine, and isonipecotic acid. Preferential inhibition of glial transport (cultured astrocytes) of [^{3}H]GABA occurred with compounds such as (3RS, 4SR)-4-hydroxynipecotic acid, guvacine, (RS)-N-methylnipecotic acid, (RS)-ß-proline and ß-alanine, and neuronal transport with (R) trans-4-amino-4-methyl crotonic acid (3RS, 4SR, 5SR)-4-hydroxy-5-methyl-nipecotic acid and (RS)-3-hydroxy-5-aminovaleric acid. Stereospecifity of the uptake and receptor sites was also apparent since these sites showed opposite stereoselectivity

for (R)- and (S)-trans-4-amino-4-methylcrotonic acid and (R)- and (S)-β-proline. Iversen and Kelly (1975) previously proposed that glial and neuronal uptake could be separated by different substrates and had shown that [^{3}H]-2,4-diaminobutyric acid (DABA) acts on neurones as an alternative substrate for the neuronal high affinity uptake of GABA but was poorly taken up by glial cells whereas [^{3}H]β-alanine was a selective substrate for the glial uptake of GABA (see also Desarmenien, Feltz and Headley, 1980).

The most potent inhibitors of GABA uptake into neuronal elements are 2-hydroxy-GABA, 2-chloro-GABA and 2- and 4-methyl-GABA (Beart and Johnston, 1973), DABA (Schon and Kelly, 1974) and nipecotic acid (Krogsgaard-Larsen and Johnston, 1975). These and other inhibitors of uptake are conformationally extended analogues of GABA. Such a conformation may be important for initial binding of GABA to its carrier (Beart, Johnston and Uhr, 1972).

Differences also occur in the potency of compounds to inhibit GABA uptake in different tissues. For example, L-DABA is a much weaker inhibitor in satellite glial cells of rat sensory ganglia than in cerebral cortex slices whereas, β-alanine is a 200 times more potent inhibitor in sensory ganglia than in cerebral cortex slices (Schon and Kelly, 1974).

Other compounds have been investigated as inhibitors of GABA uptake. Thiol reagents such as PCMP, N-ethylmaleimide and carboxypyridine disulphide inhibit GABA uptake (Phillips and Kelly, 1975). Although PCMP was the most powerful

inhibitor, its action as mentioned in the excitant amino acid section on uptake, was found to be non-selective when applied microelectrophoretically in the cat spinal cord (Curtis, Duggan and Johnston, 1970). Stropthanthidin, an aglycone structurally related to ouabain inhibits GABA uptake as do a variety of electron transport inhibitors such as juglone and chloramphenicol (Beart and Johnston, 1973).

Microelectrophoretically, DABA, (D) and (L)-nipecotic acid (Curtis, Game and Lodge, 1976; Lodge, Johnston and Curtis, 1978), 2,2-dimethyl-β-alanine (Curtis, Game and Lodge, 1976) and guvacine (Lodge, Johnston and Curtis, 1978) reversibly enhanced and prolonged the action of GABA but not that of glycine on spinal (Curtis, Game and Lodge, 1976; Lodge, Johnston and Curtis, 1978) and cerebral and cerebellar cortical neurones (Curtis, Game and Lodge, 1976). DABA also enhances the depolarization of Ia terminals by GABA (Sastry, 1979). In the spinal cord the action of β-alanine is also enhanced but taurine is unaffected. In the cerebral and cerebellar cortices both DABA and D-nipecotic acid enhance GABA only slightly more than either β-alanine or taurine. Perhaps surprisingly neither of these compounds enhanced GABA mediated basket cell inhibition of cerebellar Purkinje cells.

However, the order of potency of compounds in vivo is of a similar order to the potency of these compounds to inhibit [^{3}H]GABA uptake in vitro although the difference between compounds in vivo is much less than might be expected from the IC_{50} values obtained in vitro (Lodge, Johnston and Curtis, 1978).

2. METHODS

The experiments described in this thesis were performed on the spinal cord of anaesthetized cats. This preparation was used for the recording of extracellular action potentials of single neurones in response to microelectrophoretically-administered compounds or in response to peripheral nerve or ventral root stimulation. In addition the same preparation was used to determine the effects of microelectrophoretically-ejected substances on the excitability of single 1a primary afferent terminations and fibres stimulated in the lumbar ventral horn motor nuclei.

(a) General methods

Cats of either sex were anaesthetized with pentobarbitone sodium (40 mg kg^{-1} intraperitoneally initially, supplemented intravenously when necessary). After induction of anaesthesia, the right common carotid artery, the left cephalic vein and the trachea were cannulated. After preparation of the recording site and peripheral nerves for stimulation (see following sections) the animal was mounted in a rigid Canberra frame using thoracic, pelvic and lumbar clamps. The animal was suspended so that the abdomen and thorax were held clear of the base of the frame. This procedure which minimized respiratory influences on the spinal cord, was particularly important when recording the excitability of single primary afferent terminals. The temperature of the animal was maintained between 36 and 38°C by means of heating pads placed under the abdomen and chest. A thermometer and temperature sensitive probe were placed

between the rib cage and right scapula, the current flowing through one of the heating pads being controlled by a solid state current regulatory unit. The carotid blood pressure and pulse rate were continuously monitored in all experiments and end tidal CO_2 was recorded during experiments on primary afferent terminal excitability. A mixture of macrodex (5%) and rheomacrodex (2%) in 'normal saline' was administered intravenously when necessary to maintain blood pressure, experiments being terminated if blood pressure fell below 100mm of mercury. During the experiments carried out in chapter 7, the cat's bladders were emptied at regular intervals and as a prophylatic measure towards the control of oedema of the cord, a single intramuscular dose of dexamethosone sodium phosphate (0.5 mg kg^{-1} was administered at the time of anaesthetizing the animal.

(b) Spinal cord preparation

After separation of the exposed vertebral muscles the laminae of all lumbar vertebrae were removed and the spinal cord and dura transected at the thoraco-lumbar junction, after the localized injection of 1% procaine into this region of the spinal tissue. The dural sac was opened dorsally over the lumbar and sacral cord. Ventral roots L7 and S1 were sectioned at the intervertebral foramen and mounted on platinum electrodes which served for either stimulation or recording. Ventral roots S2 and S3 were also transected (but not mounted) during the experiments of chapter 7 so that all impulses recorded peripherally were

confined to antidromic impulses in primary afferents After mounting the animal in the frame.as previously described, a pool was formed from the skin flaps and the exposed spinal cord and vertebral muscles were covered with warmed liquid paraffin which was maintained at 36 to 38° C by heating coils.

(c) Leg preparations

In chapters 3, 4 and 6, the tibial and common peroneal nerves of the left hind limb were exposed, sectioned and mounted on bipolar platinum wire electrodes. An oil pool was formed from the skin flaps and again filled with warmed liquid paraffin. Electrical stimulation of these nerves provided field potentials which facilitated electrode placement for the recording of extracellular action potentials from single unidentified interneurones. For the experiments described in chapter 5 on the effects of antagonists on synaptic activation of identified interneurones the following nerves were used· sural, medial, gastrocnemius (MG) and lateral gastrocnemius-soleus (LG), plantaris (Pl), flexor digitorum longus (FDL), tibial and common peroneal. Medial and lateral gastrocnemius were usually combined and stimulated together.

The experiments of chapter 7 required exposure and sectioning of the posterior biceps and semi-tendinosus nerves (PBST) which were mounted together for stimulating. Pl, FDL, LG and MG were also used but in this case LG and MG were mounted for recording or stimulating separately. FDL was usually divided into two bundles for the same purposes.

(d) Microeletrophoretic techniques

The technique of microelectrophoresis was used to eject substances into the region of individual neurones or primary afferent terminals. Ionized compounds were electrically ejected into the extraneuronal space from multibarrel glass micropipettes. Seven barrel micropipette blanks were drawn out to a fine tip in a vertical electrode puller. These tips were viewed microscopically and by means of a fine glass rod, attached to a micromanipulator (de Fonbrune No. 190 or Stoelting Co. Inc. USA) the tips were broken back to a total diameter of between 5 and 8 µm. The dry micropipettes were then filled from above with centrifuged solutions and filling was completed by centrifuging the partially filled micropipette. The central barrel was filled with 3.6 M NaCl and the six outer barrels contained aqueous solutions of the substances to be tested in the particular combination required for an experiment. Details of the solutions are outlined under the appropriate sections on experimental design. In Chapter 7, single 4 M NaCl-containing electrodes, prepared in a similar fashion to the multibarrel electrodes, were used to locate motoneuronal nuclei in response to stimulation of the homonymous nerve. After filling, the micropipettes were examined microscopically using a water immersion lens and the electrical resistance of each barrel was measured. Electrodes were bevelled to give an approximately 45° angle. Micropipettes were also examined microscopically after use. The micropipettes were held in either a manually-operated or stepping-motor micromanipulator

both of which allowed movement in three axes and which were attached to the rigid Canberra frame. When the micro-electrode was placed in the spinal tissue, the resistance of each barrel was again measured. Electrical contact was made with the centre barrel by means of a small Ag/AgCl junction and with the other barrels by means of individual silver wires. An Ag/AgCl junction covered with gauze saturated with 165mM NaCl was sutured to exposed muscle near the recording site to act as the indifferent electrode (earth). The resistance of each barrel *in situ* was determined by establishing a 0.5V potential difference across each solution and measuring the resultant current flow. Retaining potentials of the order of 0.5 V were applied to each outer barrel in order to control the diffusional efflux of active ions from barrels other than those containing NaCl or KCl. During the experiments in chapter 7 electrophoretic ejecting currents were always balanced by passing an equal (ejecting + retaining) current in the opposite direction through a barrel containing NaCl (1.8 M) since changes in terminal threshold might be expected from alterations in the ionic resistance of the medium between the stimulating electrode and the terminal. Thus for example the ejection of L-glutamate (cationic retaining current 15 nA) by an anionic current of 40 nA was balanced by ejecting Na^+ with a cationic current of 55 nA. Retaining currents were generally not balanced hence the net current flow through all barrels remained constant but not zero.

(e) Experimental design

(i) Chapter 3

The effect of the isomers of AMPA and related compounds on the activity of interneurones and Renshaw cells was investigated. Extracellular recordings were made in this chapter (and chapters 4, 5 and 6) through the central 3.6 M NaCl containing barrel. The Ag/AgCl junction which made electrical connection with the recording barrel of the micropipette was connected to a negative capacitance cathode follower. After suitable amplification the spike potentials were displayed upon a double beam oscilloscope. The output of the cathode follower was also amplified and a window discriminator used to select the action potentials of one neurone which then triggered a pulse generator. This output and the spike potentials were observed simultaneously on a double beam oscilloscope and the pulses generated were counted by means of a ratemeter. The output of the ratemeter (time constant 0.2s) was displayed on a rectilinear ink-writing recorder upon which side marker pens indicated periods of microelectrophoretic ejection of compounds. There was thus a continuous display of mean cell firing frequency and this could be readily correlated with the effects of electrophoretically administered compounds.

Amino acids were administered from the outer barrels for times sufficient in most cases to obtain maximal effects at the particular rate of ejection. In view of the relatively slow onset of excitation by some amino acids, times of ejection were frequently reduced so that maximum

rates were just at the plateau level recorded with longer ejection times. Since antagonism between microelectrophoretically administered agents includes both a slower onset and reduced effect of the agonist (Curtis, 1976) the effects of excitant amino acid antagonists is also reflected (see figures 2 and 4) by a slower onset and lower maximum rate of firing. Results based on the comparison of these firing rates appeared not to differ significantly from those based on a comparison of plateau levels of firing. The approximate relative potencies of the compounds were determined from a comparison of electrophoretic currents required to produce equal but submaximal excitation of the cells. Comparison of potencies were made between compounds ejected from the same micropipette and on the same neurone.

The following solutions of compounds (unionized nomenclature) were tested: DL-quisqualic acid (5 mM in 150 mM NaCl) N-methyl-D-aspartic acid (NMDA;50mM in 150 mM NaCl, pH 7.5) DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA:100 mM, pH 7.5), L-AMPA (100 mM, pH 7.6), D-AMPA (100 mM, pH 7.3), DL-α-amino-3-hydroxy-5-bromomethyl-4-isoxazolepropionic acid(ABPA:150 mM, pH 7.3), DL-α-amino-3-hydroxy-5-tertbutyl-4-isoxazolepropionic acid (ATPA:100 mM, pH 8.0), DL-α-amino-β-methyl-3-hydroxy-4-methyl-5-isoxazolepropionic acid (AMMA:50 mM in 150 mM, pH 7.5) DL-α-amino-3-methoxy-5-methyl-4-isoxazolepropionic acid (O-methyl-AMPA:100 mM, pH 7.6), DL-2-amino-5-phosphonovaleric acid (2APV:50 mM in 150 mM NaCl, pH 7.5) and glutamic acid diethylester (GDEE:200 mM, pH 3.5).

(2) Chapter 4

The microelectrophoretic effect of a series of recently synthesized compounds on the excitation of interneurones and Renshaw cells by acidic amino acids and acetylcholine (Renshaw cells) was investigated in this study. Ejecting currents for different excitants were adjusted to give approximately equal but submaximal firing rates (30-80 spikes s^{-1}), for 10-60 sec, and antagonists were ejected (after two or three control agonist responses) to produce a 50-100% reduction of the effect of a particular excitant, with a limit of 80 nA as the maximum current used.

The following amino acid agonists and antagonists were used in the outer barrels of the micropipettes: NMDA (50 mM in 150 mM NaCl); DL-Kainic acid (5 mM in 150 mM NaCl, pH 7.5); L-glutamic acid (200 mM, pH 7.5); L-aspartic acid (200 mM, pH 7.5); DL-quisqualic acid (5 mM in 150 mM NaCl, pH 7.5); DL-cis-2,3-piperidine dicarboxylic acid (2,3 PDA; 200 mM, pH 7); DL-cis-2,5-piperidine dicarboxylic acid (2,5 PDA, 200 mM, pH 7); β-D-aspartyl-β-alanine (βDAA; 200 mM, pH 7.5), γ-D-glutamylglycine (γDGG; 200mM, pH 7.5); 2APV (50mM in 150mM NaCl, pH 7) DL-2-amino-7-phosphonoheptanoic acid (2APH, 50 mM in 150 mM NaCl, pH 7) and DL-2-amino-4-phosphonobutyric acid (2APB, 200 mM, pH 7). Acetylcholine bromide (ACh, 250 mM) was used to excite Renshaw cells.

(3) Chapter 5

Interneurones within laminae III-VI and Renshaw cells were identified by responses to afferent volleys or antidromic stimulation of ventral roots. Monosynaptic and polysynaptic excitations of interneurones were distinguished on the basis of central latencies (for impulses in lower threshold afferents) and the response to repetitive afferent stimulation (300 Hz). Stimulus intensities are indicated (see figure 12) in multiples of the threshold (T) of the lowest threshold fibres of a particular nerve and central latencies were determined using a stimulus of at least 1.5T, the afferent volley being recorded from the surface of the spinal cord close to the appropriate dorsal root entry. During this study, the 'background' rate of cell firing was generally maintained aproximately constant by the continuous ejection of DL-quisqualate, NMDA or ACh in order to preclude variations in postsynaptic excitability being responsible for changes in the response to synaptic excitation (Curtis, Lodge, Bornstein and Peet, 1981). Such a consideration was not taken into account in previous studies of this nature. The particular excitant used was selected as that found during the experiments outlined in chapter 4 to be least affected by the particular antagonist.

Histograms of the firing of individual neurons before, during and after synaptic excitation were constructed using a 128 channel Ortec 4620/4621 analyser.

All solutions of agonists or antagonists were the same as those used in chapter 4.

(4) Chapter 6

Background firing rates of cells were maintained between 20 and 40 spikes s^{-1} by the continuous ejection of either NMDA or DL-quisqualate. For the majority of cells studied, there was less than 50% reduction in the amplitude of the negative component of extracellularly recorded action potentials at firing rates of 80-100 spikes s^{-1}. Studies were not continued when excitation at lower rates resulted in complete suppression (depolarization block) of firing.

Histograms of the firing of individual neurones before, during and after the ejection of excitant were constructed using a 128 channel Ortec 4620/4621 analyser; in general the responses of three consecutive cycles were summed, bin width 1s. The outer barrels contained aqueous solutions of the following substances: L-glutamic acid (200mM, pH 7.5); D-glutamic acid (200mM, pH 7.5); DL-quisqualic acid (5 mM in 150 mM NaCl, pH 7.8); NMDA(50 mM in 150 mM NaCl, pH 7.5); L-aspartic acid (200mM, pH 7.5); D-aspartic acid (200mM, pH 7.5); bicuculline methochloride (BMC; 10 mM in 150 mM NaCl); strychnine hydrochloride (2 mM in 150 mM NaCl), γ-aminobutyric acid (GABA, 200mM, pH 3.0); glycine (500mM, pH 3.0), ACh (250mM); 2APV (50 mM in 150 mM NaCl, pH 7.1); dihydro-β-erythroidine hydrobromide (DHβE, 10 mM in 150 mM NaCl).

(5) Chapter 7

Location and identification of Group Ia terminals

Single 4 M NaCl-filled recording electrodes (1-5 M Ω) were used initially to locate Pl, FDL and gastrocnemius motoneurones in the lower L7 and S1 segments on the basis of field and action potentials evoked by stimulation of these nerves and ventral roots. These electrodes were then replaced by seven barrel micropipettes and located in a similar fashion to the single electrodes, the field and action potentials being recorded by the centre 3.6 M NaCl-filled barrel (3-6 MΩ). The recording barrels were then used as stimulating microelectrodes and negative (cathodal) 300 μs, < 2 μA pulses, used to locate sites of minimal threshold for individual group Ia fibres, impulses in which were recorded monophasically from the appropriate muscle nerve. A series of differential amplifiers and multibeam oscilloscopes were used to monitor activity in five peripheral nerve bundles simultaneously. Stimulating currents were determined by measuring the voltage across 100 Ω between the indifferent electrode attached to muscles of the back and earth, pulse shape being adjusted by a variable capacitor (10-400 pF) in parallel with a 2 MΩ limiting resistance in series with the stimulating electrode.

Generally, no or little (< 10 Hz) spontaneous activity was recorded from the muscle nerves, but occasionally continuous bursts of high frequency firing (> 50 Hz) in one or more nerves prevented further study of Ia fibres within them. Such spontaneous firing appeared to be associated

with damage to the cord roots or nerves, or with a poor general condition of the experimental animal. Fibres with velocities exceeding 70 ms^{-1} (determined using a 1.5 T stimulus) were considered as group Ia and the antidromic latencies of the evoked impulses were within 0.7 ms of the group Ia orthodromic latency (see Curtis and Lodge, 1982). Group II fibres which also terminate on motoneurons (see Fyffe, 1979), had considerably longer antidromic latencies.

Terminations were selected for further study which had electrical thresholds within the range 0.4-1.0 μA, and changes in threshold induced synaptically or by microelectrophoretically-administered agents were measured by adjusting the pulse amplitude by means of a feedback system which maintained the discharge frequency of an individual fibre at 5 Hz when stimulated at 10 Hz (Curtis, 1979).

The amplitude of the threshold pulse was plotted continuously on a pen recorder and in general two channels were used, one with a time constant of response of approximately 80 ms, the other of 700 ms and twice the gain. Instability produced by cardiovascular, respiratory or other cyclic movements were readily apparent and when such changes of threshold exceeded 2% they severely limited the interpretation of small drug-induced alterations. Changes in threshold occurring over periods of minutes produced by either movement of the electrode or possibly by leakage of pharmacologically active compounds from micropipettes were more difficult to assess. Those due to slow movement of the electrode within the tissue could frequently be compensated for by adjusting the position of the micropipette. It was

generally found that terminations with thresholds less than 0.4 μA were readily influenced by electrophoretically-administered agents, but were particularly sensitive to movement of the micropipette and that terminations with thresholds exceeding 1 μA were more stable but were relatively insensitive to low currents of electrophoretic compounds. Because of these factors tending to alter the threshold of terminations, changes induced by substances administered microelectrophoretically were considered significant only when fully reversible and readily reproducable.

Since the unmyelimited terminations of primary afferents are the probable site of PAD generation it was important to determine that these regions were within the influence of microelectrophoretically-ejected compounds. Terminal regions were identified by determination of their anodal blocking factor. It has previously been reported (Katz and Miledi, 1965) that unmyelinated axon terminals at the amphibian neuromuscular junction did not respond to pulses (500 μs duration) which were 2-3 times the amplitude of the threshold pulse. This blocking effect was ascribed to a hyperpolarization of the membrane by current flowing inwardly adjacent to the site of depolarization, inexcitability occurring due to anodic surround. The ratio (blocking factor) between threshold currents and those which suppress excitation might be expected to be lower for unmyelinated structures such as boutons and preterminal axons than for those excited at a node.

Blocking factors of eight to 10 have been reported for axons of spinal neurones (Jankowska and Roberts, 1972, Roberts and Smith, 1973) 10 for corticospinal axons (Shinoda, Arnold and Asanuma, 1976) and seven to 14 for rubrospinal axons (Shinoda, Ghez and Arnold, 1977). In a previous study from this laboratory (Curtis and Lodge, 1982) the values of the blocking factor for eight cutaneous and 10 muscle afferent fibres stimulated within the dorsal columns ranged from 3.3 to 12, with a mean of 6.8 ± 0.5 S.E.M. For 38 other dorsal column fibres the values ranged from 2.5 to 16 with a mean of 5.9. In contrast the values of 72 structures stimulated in the immediate vicinity of motoneurones and considered to be group Ia terminations on the basis of sensitivity to GABA and/or the reduction of synaptic PAD by BMC ranged from 1.2 - 6.0 with a mean of 3 ± 0.1 S.E.M. In this present study therefore pharmacological investigations were carried out on structures with anodal blocking factors of less than four.

Stimulating electrodes were assumed to be within 80µm of terminals excited by pulses of less than 1µA (Jankowska and Roberts, 1972). Considerable difficulty was experienced, however, in using terminations at the lower end of this range of anodal block values especially when the feedback device over-compensated for small increases in threshold and then failed to excite the termination. In addition, pulses as small as 0.5 µA frequently excited several terminations, the antidromic action potentials of which could not be separated on the basis of amplitude or latency.

Afferent Volley PAD

The thresholds of extensor group Ia terminations and fibres were reduced by tetanic (four volley, 320 Hz) stimulation of PBST nerve at an intensity of 1.5-3.0T. PAD for any one termination was recorded as a mean value of the changes in threshold recorded over a period of 8 s stimulation. Threshold was assessed automatically at 10 Hz and at a conditioning interval of usually 30 msec.

(3) Measurement of coupling resistances

Changes in extracellular ion concentrations can be detected by measuring alterations in the "coupling" resistance (Coombs, Eccles and Fatt, 1955) between adjacent barrels of a seven barrel micropipette. The value of this resistance presumally depends largely on the nature of the medium (extracellular fluid, damaged neurones and glia) in the immediate vicinity of the barrel orifices, the region most influenced by substances ejected from other barrels of the micropipette. Coupling resistances were derived from potentials recorded by the centre barrel (3.6 M NaCl: 3-6 MΩ) of seven barrel micropipettes located near motoneurones during the passage of 10 or 20 μA current pulses through another barrel which contained 1.8 M NaCl (5-15 MΩ). These potentials were measured after the initial capacitative artefact had subsided and were either averaged (50 ms pulses, 5 Hz, 16-64 sweeps) or plotted continuously (500 ms, 1 Hz) on a paper recorder (Offner Dynograph). This latter method also allowed continuous monitoring of changes in the junction potential at the tip of the recording electrode.

When measured with the tips of the micropipettes (4-6 µm overall diameter) immersed in 165mM NaCl, coupling resistances for seven barrel micropipettes were found to range from between 20 and 50 KΩ and were not significantly altered during the balanced electrophoretic administration of Na^+, K^+ or amino acids from other barrels using currents as high as 100 nA. Dilution of the external NaCl solution over the range 160-100mM increased coupling resistances by 20-25% in a linear fashion, the increase being higher for micropipettes having coupling resistances of 40-50 KΩ (165mM NaCl) than for those of lower values.

Within the spinal tissue in vivo, coupling resistances were considerably higher (40-800 KΩ) and percentage alterations may have been provided by smaller changes in extracellular ion concentrations than under in vitro conditions. Measurements required considerable stability of the position of the micropipettes within the tissue and of the resistances of the recording electrodes (see Coombs, Eccles and Fatt, 1955) and thus, as for measurement of threshold changes, alterations in coupling resistance associated with the microelectrophoretic ejection of amino acids in regions near motoneurones were only considered significant when reproducable and fully reversible.

Microelectrophoretic methods

Thresholds of terminals were occasionally altered by the balanced ejection of Na^+ and Cl^- (usually to 60 nA). Changes in threshold greater than 5% excluded a fibre from

further investigation. Although balanced ejections of Na^+ and K^+ (40-80 nA) occasionally had no effect on coupling resistances, more frequently the values were rapidly and reversibly increased 10-20% by K^+ and similarly decreased by Na^+. As a consequence, amino acids were tested by ejecting from solutions undiluted with NaCl, using electrophoretic currents to produce peri-electrode concentrations comparable to those found to produce alterations in termination thresholds.

The following solutions were used in the barrels of the micropipettes: GABA (100mM, pH 3, HCl); L-glutamic acid (200mM, pH 7.5, NaOH); BMC (10mM in 150mM NaCl); muscimol (50mM in 150mM NaCl, or 100mM, pH 3, HCl); dihyromuscimol (50mM, pH 3, HCl); piperidine-4-sulphonic acid (P4S, 50mM in 150mM NaCl or 100mM, pH 3, HCl); 3-aminopropane sulphonic acid (3APS; 50mM in 150mM NaCl or 100mM, pH 3); isoguvacine hydrobromide (50mM in 150mM NaCl, pH 3); isonipecotic acid (100mM, pH 3, HCl); kojic amine (100mM, pH 3.5, HCl); 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol (THIP; 100mM, pH 3, HBr); DL-3-hydroxy-GABA (100mM, pH 3, HCl), DL-2-chloro-GABA (100mM, pH 3, HCl); trans-4-aminocrotonic acid (100mM, pH 3.2, HCl); 4-methyl-aminobutryic acid (100mM, pH 3.4, HCl); 4-aminovaleric acid (100mM, pH 3, HCl); DL-2-hydroxy-GABA (100mM, pH 3, HCl); D-nipecotic acid (NIP; 100mM, 200mM or 5mM in 150mM NaCl, pH 3, HCl); imidazole acetic acid (100mM, pH 3, HCl); glycine (500mM, pH 3, HCl); β-alanine (500mM, pH 3, HCl); L-histidine (100mM, pH 3.9, HCl), taurine (100mM, pH 8, NaOH); L-2,4-diaminobutyric acid

(DABA 100mM, pH 3, HCl) 2.2-dimethyl-β-alanine (DMβA 100mM, pH 3.3, HCl), L-ornithine (100mM, pH 3, HCl), L-lysine (100mM, pH 3, HCl); L-2,3-diamino propionic acid (DAPA 100mM, pH 3.1, HCl); L-arginine (100mM, pH 3. HCl) noradrenaline (NA; 100mM, pH 3.4, bitartrate) 5-hydroxy-tryptamine-creatinine sulphate (5HT; 50mM, pH 3.8) and ouabain (see text).

As with the excitant amino acid experiments described in this thesis, potencies were related to the current required to produce equal effects, making allowance for the dilution of some of the amino acids in 150mM NaCl rather than in water.

(f) Sources of drugs and chemicals

AMPA and related compounds were supplied by Dr.P. Krogsgaard-Larsen, the amino acid antagonists used in chapters 4 and 5 by Dr. J.C. Watkins and kojic amine by Dr. G.G. Yarbrough. Other compounds used in this thesis have previously been synthesized by Drs. P. Krogsgaard-Larsen, J.C. Watkins and G.A.R. Johnston or were obtained from commercial sources.

(3) GLUTAMIC ACID AGONISTS: STEREOCHEMICAL AND CONFORMATIONAL STUDIES OF AMPA AND RELATED COMPOUNDS.

It was considered in the General Introduction that because of its conformational flexibility L-glutamate was capable of interaction with its own receptor in an extended form and with the L-aspartate receptor in a folded conformation (Van Gelder, 1971). Such a suggestion was supported by investigations into the sensitivities of a series of amino acid analogues to the antagonists GDEE and DαAA which showed that although micro-electrophoretic excitation by DL-quisqualate was selectively antagonized by GDEE and that to NMDA by DαAA, excitations by L-aspartate and L-glutamate were relatively sensitive to both antagonists (Hicks, Hall and McLennan, 1978; Davies and Watkins, 1979).

These studies have therefore led to the classification of aspartate and glutamate receptors with reference to their potent and selectively antagonized analogues. However, doubt has been expressed on the suitability of DL-quisqualate for the identification and pharmacological characterization of central glutamate receptors (Krogsgaard-Larsen, Honoré, Hansen, Curtis and Lodge, 1981). This reservation has been based on observations that DL-quisqualate (as well as other conformationally-restricted glutamate analogues such as ibotenate and kainate) does not interfere with the high affinity binding of L-[^{3}H]glutamate to rat brain membranes (Biziére, Thompson and Coyle, 1980) and that DL-quisqualate is capable of effectively displacing DL-[^{3}H]kainate from high affinity binding sites in rat brain slices (Johnston, Kennedy and Twitchin, 1979).

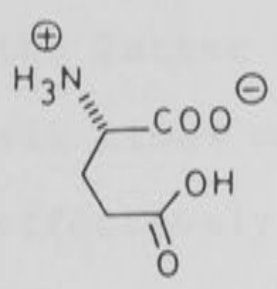
In an attempt to provide more selective agonists for both in vivo and in vitro characterization of central glutamate receptors systematic structural manipulations (Krogsgaard-Larsen, Honoré, Hansen, Curtis and Lodge, 1980, 1981) of the DαAA-sensitive ibotenate molecule (McLennan and Lodge, 1979) have been performed. Elongation of the side chain of ibotenate by one methylene group produced homoibotenate, a compound that while having a lower potency than the parent molecule was more selectively antagonized by GDEE. Homoibotenate, and the further homologues namely (2)homoibotenate and (3)homoibotenate are conformationally-restricted analogues of αAA, αAP and αAS, respectively. It is known that the D-isomers of these latter compounds have NMDA-antagonistic activity (Davies and Watkins, 1979) as do the racemic mixtures of (2)homoibotenate and (3)homoibotenate (Krogsgaard-Larsen, Honoré, Hansen, Curtis and Lodge, 1981) and the ω-phosphonate analogues of certain α-aminodicarboxylates (see Chapter 4). However, the L-isomers of the α-aminodicarboxylates have excitant properties (Evans, Francis, Hunt, Oakes and Watkins, 1979) and it would be interesting to compare the antagonistic potency of the D-isomers of the α-aminodicarboxylates and these conformationally-restricted analogues.

Although methylation of ibotenate in the four position of the isoxazole ring reduced the potency of ibotenate (but did not affect its antagonism by DαAA) methylation of homoibotenate increased its potency. Bromination on the same ring position of homoibotenate produced one of the most potent GDEE-sensitive excitants to date. Excitation by these compounds was not

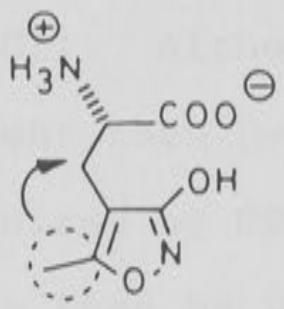
followed by the characteristic depression seen after DL-ibotenate (MacDonald and Nistri, 1977, 1978) a depression which had been attributed to in vivo decarboxylation of ibotenate to the potent GABA analogue, muscimol (Curtis, Lodge and McLennan, 1979).

These studies also produced, by alteration of the ibotenate side chain and placement at position four on the isoxazole ring, the very potent and GDEE-sensitive analogue AMPA, and the DαAA sensitive aspartate analogue, AMAA.

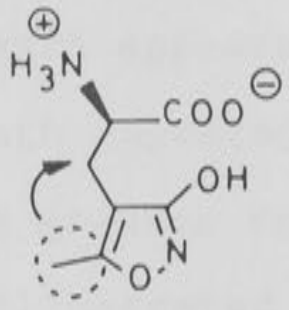
More recently, the stereoisomers of AMPA (Hansen, Lauridsen, Nielsen and Krogsgaard-Larsen, in press) have been resolved and a series of compounds structurally related to AMPA have been synthesised (Figure 1: Lauridsen, Honoré and Krogsgaard-Larsen, in preparation) and their actions on spinal neurones will be investigated in this study. These include DL-α-amino-3-hydroxy-5-bromomethyl-4-isoxazolepropionate (ABPA) and DL-α-amino-3-hydroxy-5-tertbutyl-4-isoxazolepropionate (ATPA) in which the methyl group of AMPA was replaced by more bulky substituents, DL-α-amino-β-methyl-3-hydroxy-4-methyl-5-ixozolepropionate (AMMA), derived by introduction of a methyl group into the side chain of the GDEE-sensitive glutamate agonist 4MHI and DL-α-amino-3-methoxy-5-methyl-4-isoxazolepropionate (O-methyl-AMPA) formed by methylation of AMPA on the three position of the isoxazole ring.



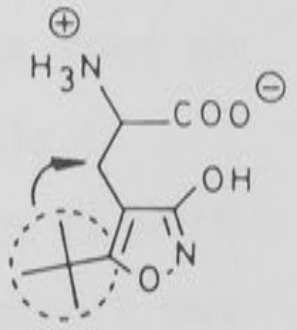
L-Glutamic acid



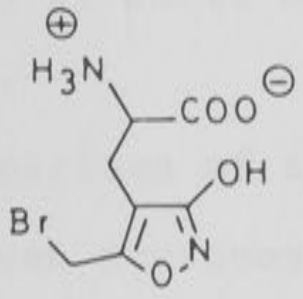
L-AMPA



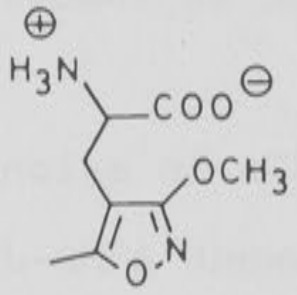
D-AMPA



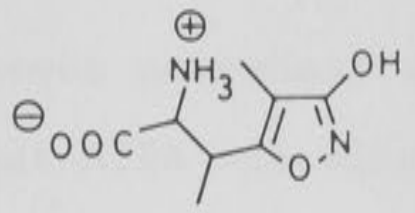
ATPA



ABPA



O-Me-AMPA



AMMA

Figure 1. The structures of L-glutamic acid, L- and D-AMPA, and some AMPA analogues. The steric effects of the alkyl groups of L- and D-AMPA and of ATPA on the conformations of the amino acid side chain of these compounds are illustrated.

RESULTS

On all seven cells tested L-AMPA proved to be a more effective excitant than D-AMPA its potency being similar to that of DL-quisqualate (taking into consideration dilution of the latter with NaCl). Although L-AMPA appeared to be four to six times more potent than D-AMPA both appeared equally effectively antagonized by GDEE: the greater reduction by GDEE of excitation by L- than by D-AMPA illustrated in Figure 2 was not observed with the other neurones investigated. As shown in Figure 3 where excitation by NMDA was completely and reversibly abolished by 2APV, neither isomer of AMPA or DL-quisqualate was affected.

Comparison of the potencies of ATPA (seven neurones) and ABPA (seven neurones) with L-AMPA showed that ATPA was slightly less potent than L-AMPA but that ABPA was considerably weaker than L-AMPA. Excitation by both ATPA and ABPA, however, was antagonized by GDEE (see Figure 4). AMMA was a weak excitant (six neurones) and merely enhanced excitation by L-AMPA, QUIS and NMDA. O-Methyl-AMPA, a neutral amino acid, showed no antagonistic or agonistic activity (three neurones).

DISCUSSION

This study has shown that both stereoisomers of AMPA are capable of potent GDEE-sensitive excitation of spinal interneurones. The difference in potency between L- and D-AMPA was perhaps unexpected in view of the only slightly greater potency of the isomers of glutamate, aspartate and cysteate (Curtis and Watkins, 1963). Such a difference in potency is

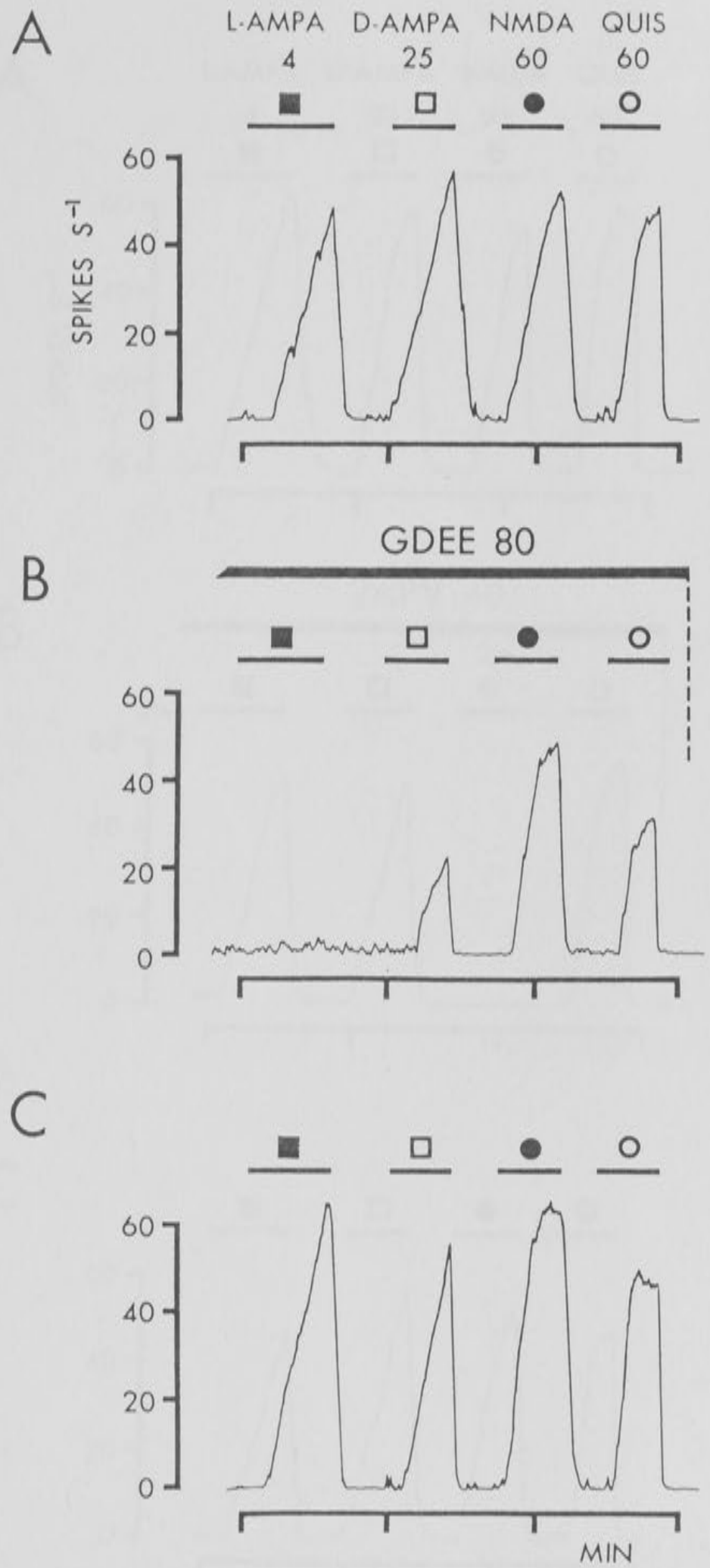


FIGURE 2 Effects of GDEE on the firing of a spinal interneurone by amino acids ejected with the indicated currents (nA) over the periods of the horizontal bars. A: before; B: during GDEE (80 nA); C: 3 minutes after GDEE.

Ordinates: firing rate, spikes s^{-1}.

Abscissae: time, min.

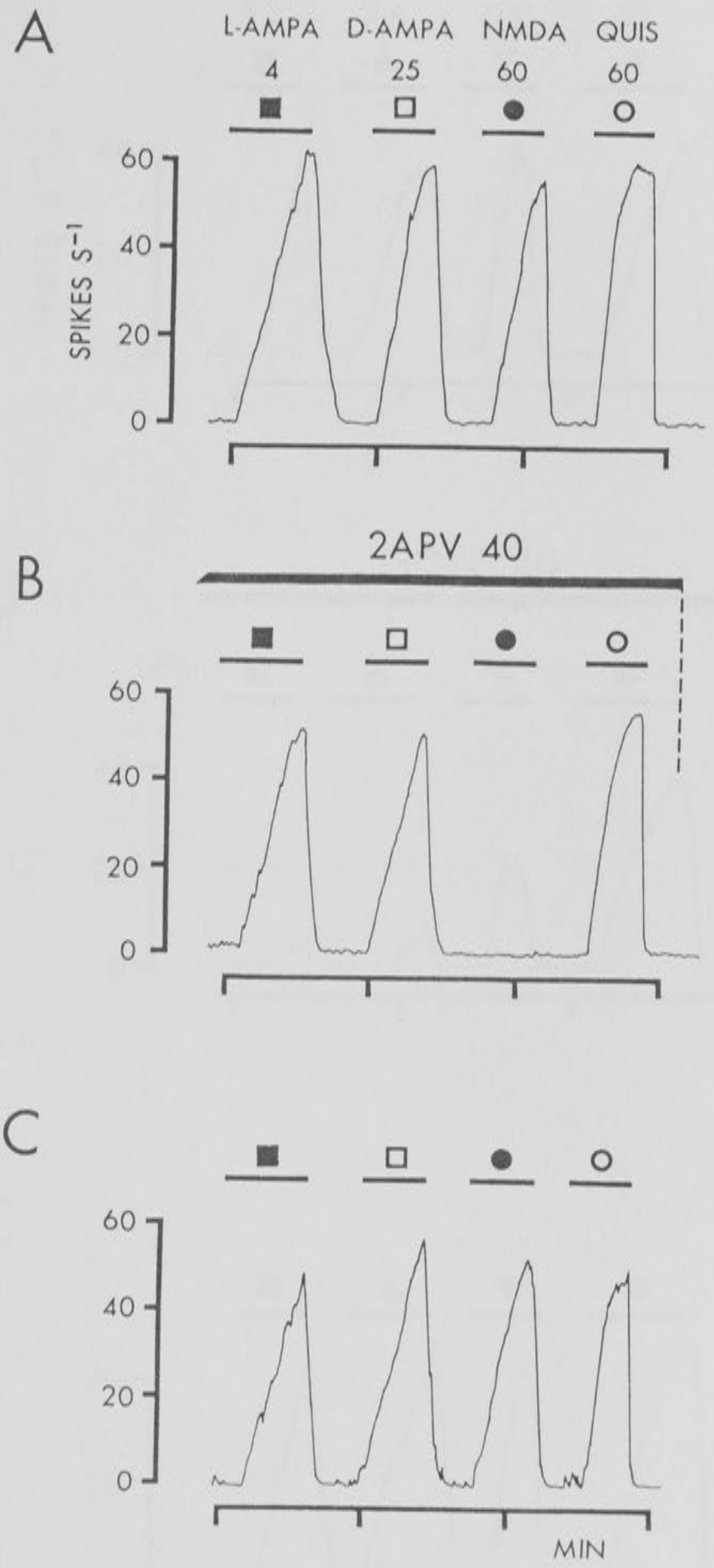


FIGURE 3 Effects of 2APV on the firing of a spinal interneurone by amino acids. A: before; B: during 2APV; C: 6.5 min after 2APV. Ordinates and Abscissae: as for Figure 2.

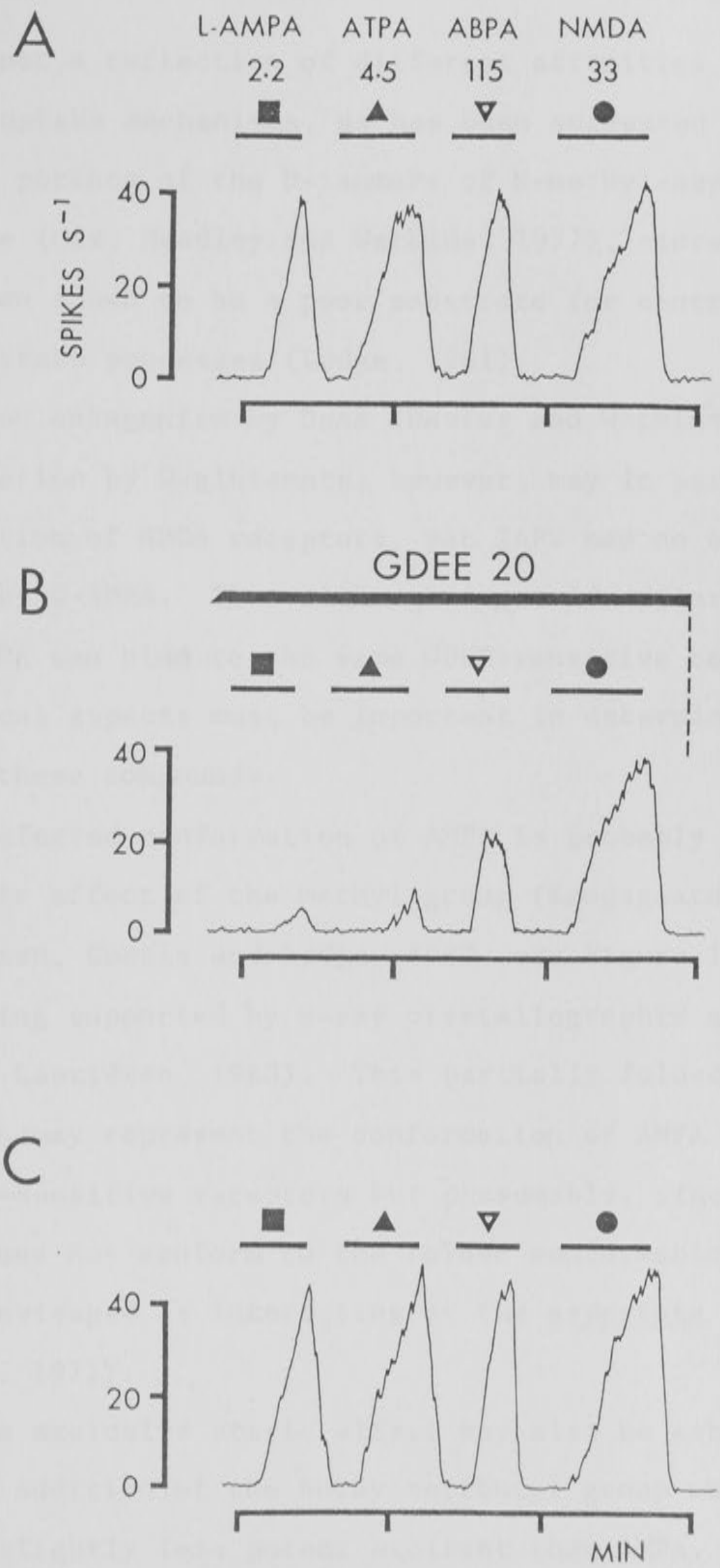


FIGURE 4 Effects of GDEE on the firing of a spinal interneurone by amino acids. A: before; B: during GDEE (20 nA); C: immediately after GDEE. Ordinates and Abscissae: as for Figure 2.

presumably not a reflection of different affinities for the amino acid uptake mechanisms, as has been suggested to explain the greater potency of the D-isomers of N-methyl-aspartate and homocysteate (Cox, Headley and Watkins, 1977), since racemic AMPA has been shown to be a poor substrate for central glutamate uptake processes (Lodge, 1981).

Based on antagonism by DαAA (Davies and Watkins, 1979) and 2APV, excitation by D-glutamate, however, may in part result from activation of NMDA receptors, yet 2APV had no effect on excitation by D-AMPA. Thus although it would appear that both D- and L-AMPA can bind to the same GDEE-sensitive receptor, stereochemical aspects must be important in determining the potency of these compounds.

The preferred conformation of AMPA is probably determined by the steric effect of the methyl group (Krogsgaard-Larsen, Honoré, Hansen, Curtis and Lodge, 1980 see Figure 1), this proposal being supported by x-ray crystallographic analysis (Honoré and Lauridsen, 1980). This partially folded conformation may represent the conformation of AMPA active at spinal GDEE-sensitive receptors but presumably, since 2APV was inactive, does not conform to the folded conformation of glutamate envisaged as interacting at the aspartate receptor (Van Gelder, 1971).

Such an exclusive steric effect may also be achieved in ATPA by the addition of the bulky tertbutyl group which produced a slightly less potent excitant than AMPA. However, the bromomethyl group of ABPA which is situated similarly to the tertbutyl group may not be purely steric and repellent. Electrostatic effects of the bromomethyl group may influence the amino acid side chain in the opposite direction. An

increased conformational flexibility of the side chain of this molecule may have the effect of preventing the molecule from consistently attaining the correct conformational characteristics for interaction with the glutamate receptor. It is of interest to note that at comparable levels of excitation this compound was less susceptible to antagonism by GDEE as compared with L-AMPA or ATPA.

AMMA was shown, presumably because of a subthreshold depolarizing action, merely to enhance the responses to other excitant amino acids. O-Methyl-AMPA showed no excitatory or antagonistic activity. Since it is known that conversion of the acidic or basic groups of the glutamate molecule to esters or amides reduces or abolishes excitatory activity as does substitutions within the intermediate carbon chain or upon the amino group (Curtis and Watkins, 1965) these latter results might have been expected. This lack of excitatory ability suggests that a necessary, but not sufficient, condition for interaction of the present class of glutamate analogues with presumed glutamate receptors is the presence of an acidic group in the position equivalent with the ω-position of the glutamate molecule. Such a proposal is supported by the results of earlier studies (Curtis and Watkins, 1965) which showed that ω-phosphonic and ω-boronic analogues of the excitant amino acids are weak, inactive or in fact have antagonistic properties (see Chapters 4 and 5).

The observations of this study also suggest that the alkyl group in the five position of the isoxazole ring of AMPA

analogues forces the molecules to adopt partially-folded conformations recognizable by glutamate receptors. Such a conformation, however, apparently prevents the uptake of AMPA by the glutamate uptake mechanism since the excitation by DL-AMPA and 4MHI, in contrast to that by DL-quisqualate and L-glutamate, is not enhanced by the amino acid uptake inhibitors DL-dihydrokainate and threo-hydroxy-D-aspartate (Lodge, 1981). Also from ratemeter records and from histograms of the firing of spinal neurones in response to their electrophoretic administration, recovery from excitation by 4MHI and DL-AMPA is longer than for DL-quisqualate and L-glutamate.

Thus these results would suggest that the excitation by AMPA and related compounds of interneurones is mediated through a GDEE-sensitive, presumably glutamate receptor. Evidence against this suggestion exists however since AMPA has been found to be virtually inactive as a displacer of L-[^{3}H]glutamate binding to rat cerebellar membranes (Honoré, Lauridsen and Krogsgaard-Larsen, 1981) although it was also shown in this study that the microelectrophoretic potency of a series of AMPA analogues for excitation of interneurones was different from their order of potency as inhibitors of L-[^{3}H]glutamate binding in vitro. This might suggest that the receptor to which L-[^{3}H] glutamate binds in vitro is not a physiological glutamate receptor, a suggestion that is supported by observations on the minimal effect of GDEE and HA-966 in displacing L-[^{3}H]-glutamate binding in vitro (Logan

and Snyder, 1972). Further studies with [^{3}H]AMPA (Honoré, Lauridsen and Krogsgaard-Larsen, 1982) in rat brain membranes have shown that a series of amino acid analogues were inhibitors of [^{3}H]-AMPA binding with the same order of potency as that shown by the excitatory action in vivo. This therefore suggests that [^{3}H]AMPA might in fact bind to an excitatory glutamate receptor and since this compound has been shown not to interfere with [^{3}H]kainate binding (Krogsgaard-Larsen, Honoré, Hansen, Curtis and Lodge, 1980) may provide a more selective agonist for the investigation of central glutamate receptors.

(4) CLASSIFICATION OF RECEPTORS FOR EXCITANT AMINO ACIDS

In the General Introduction some of the earlier studies on the use of antagonists to classify amino acid receptors into aspartate preferring (characterized by NMDA) or glutamate preferring (characterized by quisqualate) were discussed.

This chapter will consider the results obtained with recently synthesized antagonists and present data to support those obtained in previous studies suggesting the existence of a separate receptor for kainate.

Some support for differences between the kainate and quisqualate receptors comes from a recent study using the dipeptide γ-D-glutamylglycine (γDGG: Davies and Watkins, 1981) which demonstrated clear antagonism of DL-kainate excitation compared to DL-quisqualate-induced responses. Concomitant with depression of DL-kainate responses, however, was the blockade of those induced by NMDA.

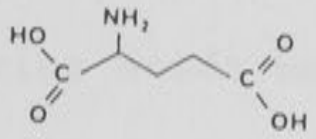
Some of these new compounds have been shown to have less specific antagonistic patterns. For example, Davies, Evans, Francis, Jones and Watkins (1981) demonstrated on neurones in the cat spinal cord *in vivo* and the frog and immature rat spinal cord *in vitro* that DL-*cis*-2,3-piperidine dicarboxylate (2,3PDA), although selectively antagonizing amino acid-induced excitation compared to that by other putative transmitters, such as acetylcholine, showed no selectivity between DL-quisqualate-, DL-kainate- and NMDA-induced excitations. These results have been partially supported by the studies of McLennan and Liu (1981) who reported that NMDA- and DL-kainate-evoked excitations of dorsal horn neurones in the spinal cord

of urethane-anaesthetized rats were concomitantly reduced by 2,3PDA but that excitation by L-glutamate was almost insensitive to this compound. In addition DL-cis-2,5-piperidine dicarboxylate (2,5PDA) in contrast to 2,3PDA was very effective as an L-glutamate antagonist and was much less active against other excitants. A similar compound DL-cis-2,6-piperidine dicarboxylate was weakly active as an antagonist but showed no pronounced selectivity of action.

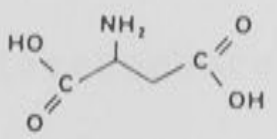
The series of ω-phosphonate analogues of α-aminodicarboxylates now extends from 2 amino-3-phosphonopropionate to 2-amino-8-phosphono-octanoate (2APO). Studies with 2APV, the phosphonate analogue of α-AA, in rat spinal cord (McLennan and Liu, 1981), cat spinal cord (Davies, Francis, Jones and Watkins, 1980) and isolated spinal cord preparations of the frog and immature rat (Evans, Francis, Jones, Smith and Watkins, 1982) have shown it to be the most selective and potent NMDA antagonist yet tested. A more elongated analogue, 2-amino-7-phosphonoheptanoate (2APH), the phosphonate analogue of α-AS, produced similar selectivity in the rat spinal cord (McLennan, 1982) and rat cortical neurons (Perkins, Stone, Collins and Curry, 1981) in vivo and the rat and frog spinal cord preparations in vitro (Evans, Francis, Jones, Smith and Watkins, 1982). The clear selectivity of these compounds towards NMDA (and L-aspartate) responses as compared to excitation by either DL-kainate or DL-quisqualate (and L-glutamate) supports the earlier classification of aspartate-preferring and glutamate-preferring receptors and also identifies differences between the NMDA receptor class on the one hand and the kainate class on the other.

In contrast to the selectivity and potency of 2APV and 2APH, studies in the cat (Watkins, Curtis and Brand, 1977) have shown that DL-2-amino-3-phosphonopropionate and DL-2-amino-4-phosphonobutyrate (2APB) were ineffective as antagonists of excitation evoked by L-glutamate, L-aspartate or DL-homocysteate, showing occasional weak excitation of dorsal horn interneurones or Renshaw cells in the cat. However, 2APB interacts, perhaps competitively, with the postsynaptic glutamate receptor of locust muscle (Cull-Candy, Donnelan, James and Hunt, 1976). Other members of this series, namely DL-2-amino-6-phosphonohexanoate (2APHEX) and 2APO are also less effective than 2APV as NMDA antagonists (Evans, Francis, Jones, Smith and Watkins, 1982) although McLennan and Liu (1981,) reported that 2APHEX was equipotent with 2APV at antagonizing NMDA responses on rat spinal neurones.

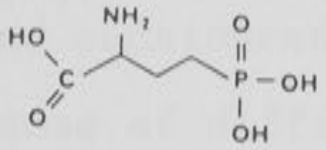
The action of the earlier NMDA antagonists was shown to be stereospecific since the antagonistic activity of αAA for example, was reported (Hall, Hicks, McLennan, Richardson and Wheal, 1979) to reside in the D-isomer while the L-isomer had weak excitatory action. As might have been expected, the antagonistic activity of 2APV (Davies and Watkins, 1982) and 2APH (McLennan, 1982) also resides in the D-isomer. The L-isomers unlike LαAA, also show weak NMDA blocking activity which is similar in character to the D-isomer although this may be due to a small residuum of the D-isomer in the samples used. D-2-amino-4-phosphonobutyrate has been shown to be a weak and relatively non-selective antagonist of amino acid-induced responses. In contrast L-2-amino-4-phosphonobutyrate either had no effect or, at higher concentrations, enhanced these responses (Davies and Watkins,(1981a).



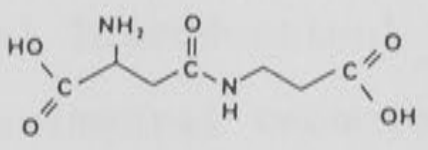
glutamic acid



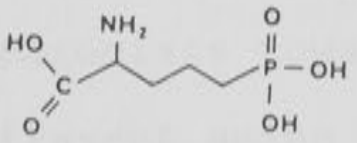
aspartic acid



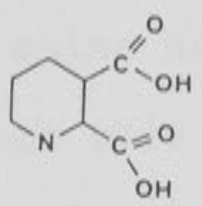
2-amino-4-phosphonobutyric acid
(2APB)



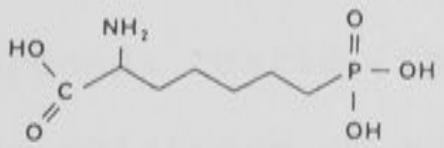
β-D-aspartyl β-alanine
(βDAA)



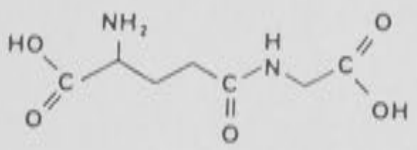
2-amino-5-phosphonovaleric acid
(2APV)



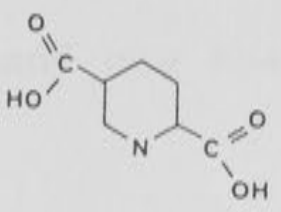
cis-2,3-piperidine dicarboxylic acid
(2,3-PDA)



2-amino-7-phosphonoheptanoic acid
(2APH)



γ-D-glutamylglycine
(γDGG)



cis-2,5-piperidine dicarboxylic acid
(2,5-PDA)

FIGURE 5 The compounds used in this study were the racemic mixtures of the cyclic aspartate analogues, cis-2,3-piperidine dicarboxylic acid and cis-2,5-piperidine dicarboxylic acid, the ω-phosphonic acid analogues, 2-amino-4-phosphonobutyric acid, 2-amino-5-phosphonovaleric acid and 2-amino-7-phosphonoheptanoic acid and the D-isomers of the monoamino alkane dicarboxylic acid-related analogues γ-glutamylglycine and β-aspartyl-β-alanine.

The structures of glutamic acid and aspartate acid are shown for comparison.

The concept of three different classes of receptor for excitant amino acids based on microelectrophoretic studies has been difficult to accept unchallenged since some of the inconsistencies in the studies on these compounds (as just outlined and considered in the General Introduction) have arisen because of differences in experimental technique, the use of different vertebrate species and regions of the CNS and the lack of agreement regarding the selectivity of some of the earlier antagonists towards the excitation of neurones in vivo both by different amino acids and by non amino acid excitants such as acetylcholine. Therefore, although this study will in some respects be of a confirmatory nature with regard to some of the compounds tested, the interpretation of this data to suggest the existence of separate sub-classes of excitant amino acid receptors should be more acceptable because of the use of similar techniques in the one tissue and one species.

RESULTS

The compounds used in this study are shown in Figure 5. The effect of each antagonist on excitations by DL-kainate, NMDA and DL-quisqualate (or L-glutamate for 2APV and 2APH) is shown for a representative neurone in Figures 6,7 and 8 and summarized graphically for all cells with that antagonist in Figures 9 and 10. The results for L-aspartate and L-glutamate have been omitted from these latter graphs for clarity but the effect of four of the antagonists on excitations by these compounds is shown in Figure 11. The results for the relative potencies of the antagonists are summarized in Table 1. The number of arrows approximates the degree of antagonism of

TABLE 1: EFFECTS OF ANTAGONISTS ON AMINO ACID AND SYNAPTIC EXCITATION OF SPINAL NEURONES

AGONIST	2,3PDA	2,5PDA	γDGG	βDAA	2APB	2APV	2APH
N-METHYL-D-ASPARTATE	++ (21)	(+) (9)	+++ (6)	+++ (7)	0 (7)	+++++ (26)	++++ (19)
L-ASPARTATE	++ (8)	(+) (3)	++ (5)	++ (5)	-	+++ (6)	++ (8)
POLYSYNAPTIC EXCITATION	++ (11)	0 (2)	++ (4)	++ (4)	(+) (6)	+++ (16)	+++ (5)
QUISQUALATE	+ (31)	(+) (12)	+ (15)	+ (16)	0 (7)	0 (11)	(+) (8)
L-GLUTAMATE	(+) (18)	0 (3)	+ (7)	+ (9)	-	+ (10)	+ (8)
KAINATE	+++ (38)	+ (12)	++ (18)	++ (20)	+ (7)	+ (15)	+ (11)
MONOSYNAPTIC EXCITATION	0 (11)	0 (2)	0 (4)	0 (4)	0 (6)	0 (16)	0 (5)
ACETYLCHOLINE	-	-	0 (1)	+ (6)	-	0 (4)	(+) (4)

agonist responses by similar concentrations (currents) of each antagonist, relative to 2APV (five arrows) making allowance for dilution of some with NaCl. Enclosure of an arrow within brackets indicates a reduced value, zero (0) no effect, - not tested, the number of cells tested is indicated in brackets.

(a) Effect of 2,3PDA and 2,5PDA on amino acid-induced excitation.

The effects of these two antagonists on excitation of spinal neurones is shown in Figure 6. With most cells, currents of 10-20 nA of 2,3PDA were sufficient to reversibly depress DL-kainate excitation by more than 50% with excitations by NMDA being slightly less sensitive and that by DL-quisqualate even less affected. With nine of 12 cells, 2,5PDA also reduced excitation by DL-kainate but at currents up to eight times higher than those for comparable effects by 2,3PDA. Responses to DL-quisqualate and NMDA were less affected at currents producing 50% reduction of excitation by DL-kainate. The effect of 2,3PDA and 2,5PDA on all cells is shown graphically in Figure 9. Recovery from these compounds was rapid and occurred within 1 min of terminating their electrophoretic ejection.

(b) Dipeptide analogues as amino acid antagonists

Both γDGG and βDAA had similar effects. Figure 7 shows the greater reduction by these compounds of the excitation of two dorsal horn interneurones by NMDA and DL-kainate, the excitation by NMDA being most reduced. In the neurone illustrated in Figure 7A-C, γDGG at 5 nA produced minimal

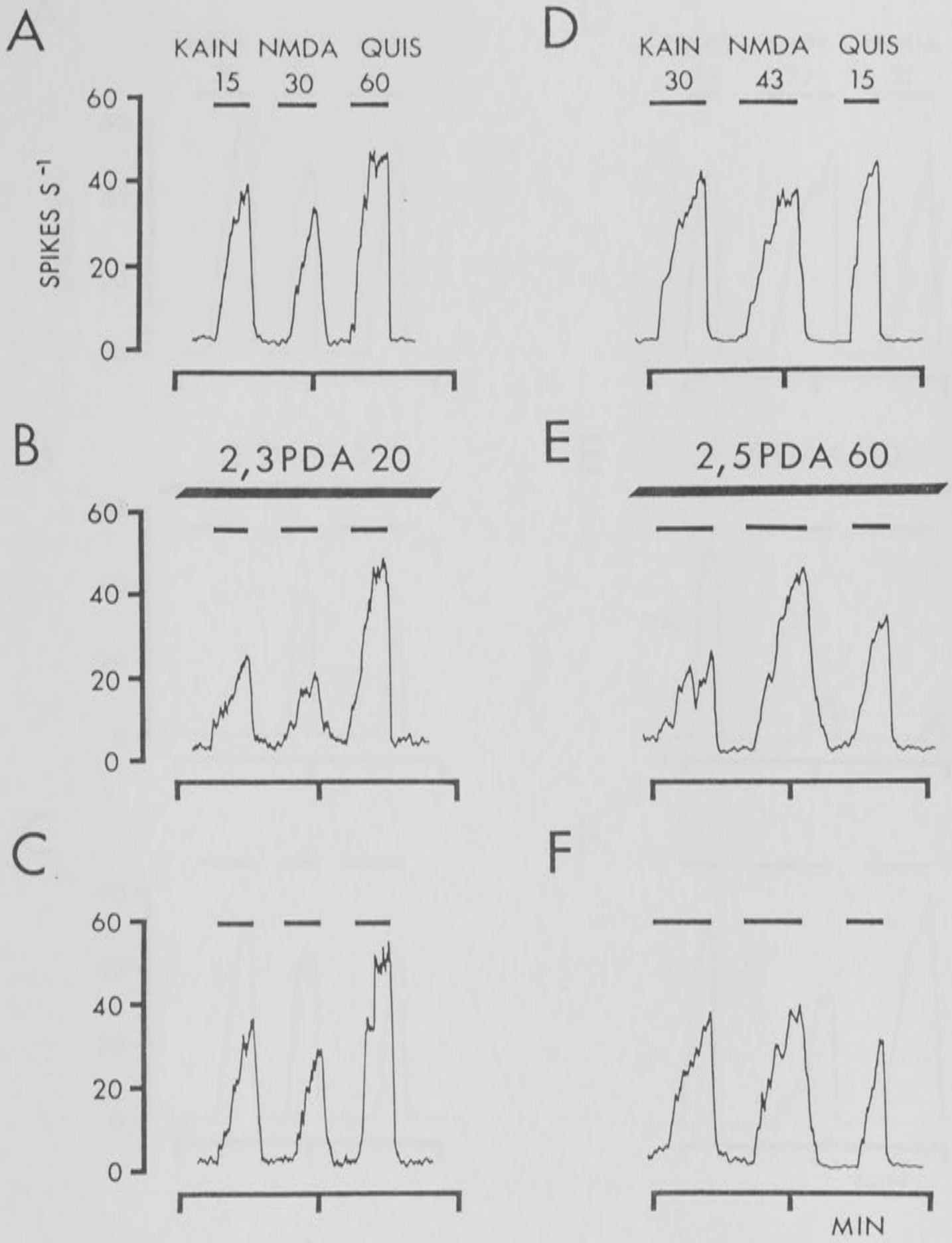


FIGURE 6 Effects of 2,3PDA (A-C) and 2,5PDA (D-F) on the excitation of a Renshaw cell and spinal interneurone respectively by DL-kainate (KAIN), NMDA and DL-quisqualate (QUIS). In this and Figures 7 and 8, periods of microelectrophoretic ejection are marked by horizontal bars, with the figure indicating current (nA). A: before; B: during 2,3PDA (20 nA); C: 2 min after terminating 2,3PDA. D: before; E: during 2,5PDA (60 nA); F: 1 min after 2,5PDA. Ordinates: firing rate, spikes per sec. Abscissae: time, min.

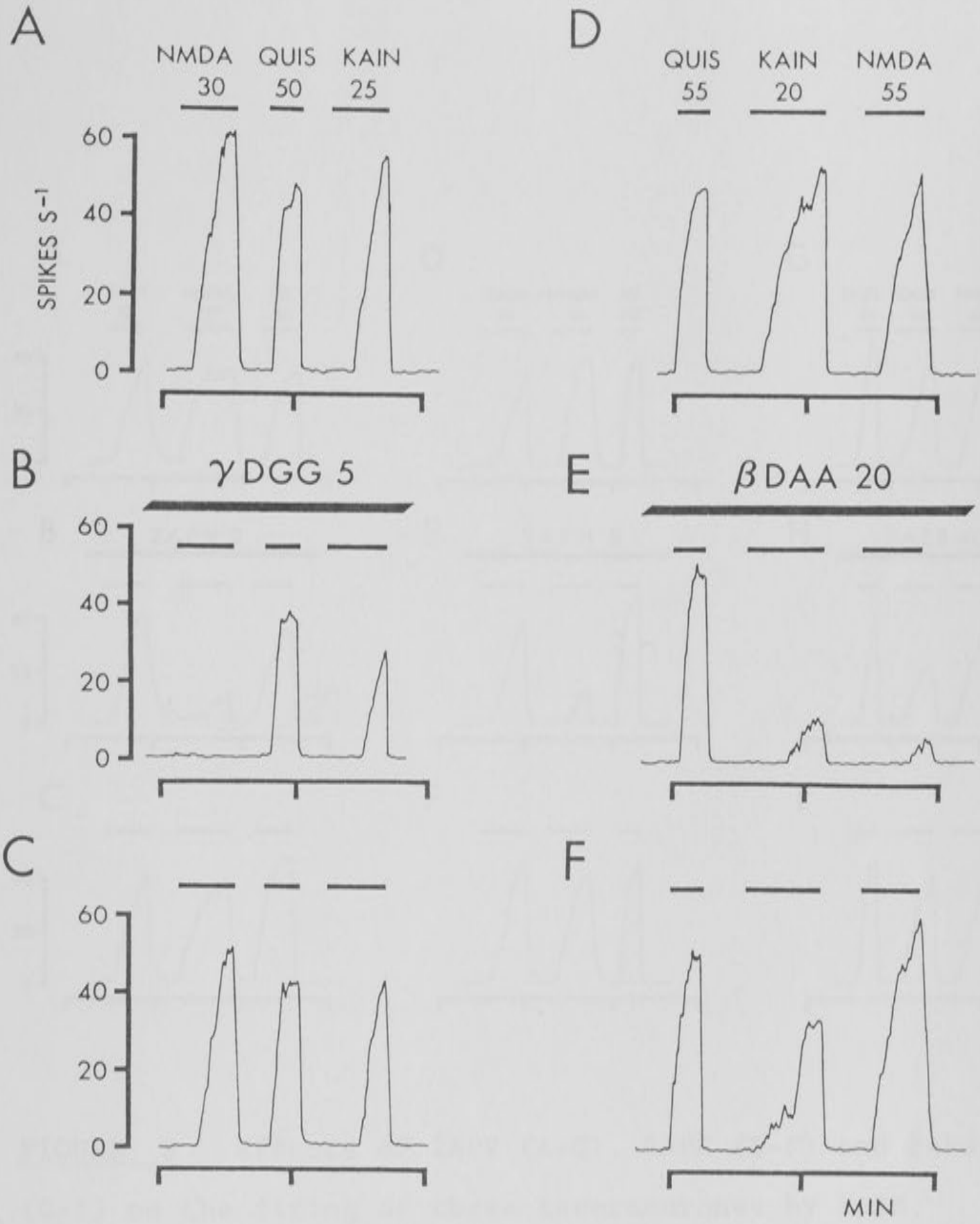


FIGURE 7 Effects of γDGG (A-C) and βDAA (D-F) on the excitation of two interneurones by QUIS, KAIN and NMDA. A: before; B: during γDGG (5 nA); C: 5 min after γDGG. D: before; E: during βDAA (20 nA); F: 4 min after βDAA.

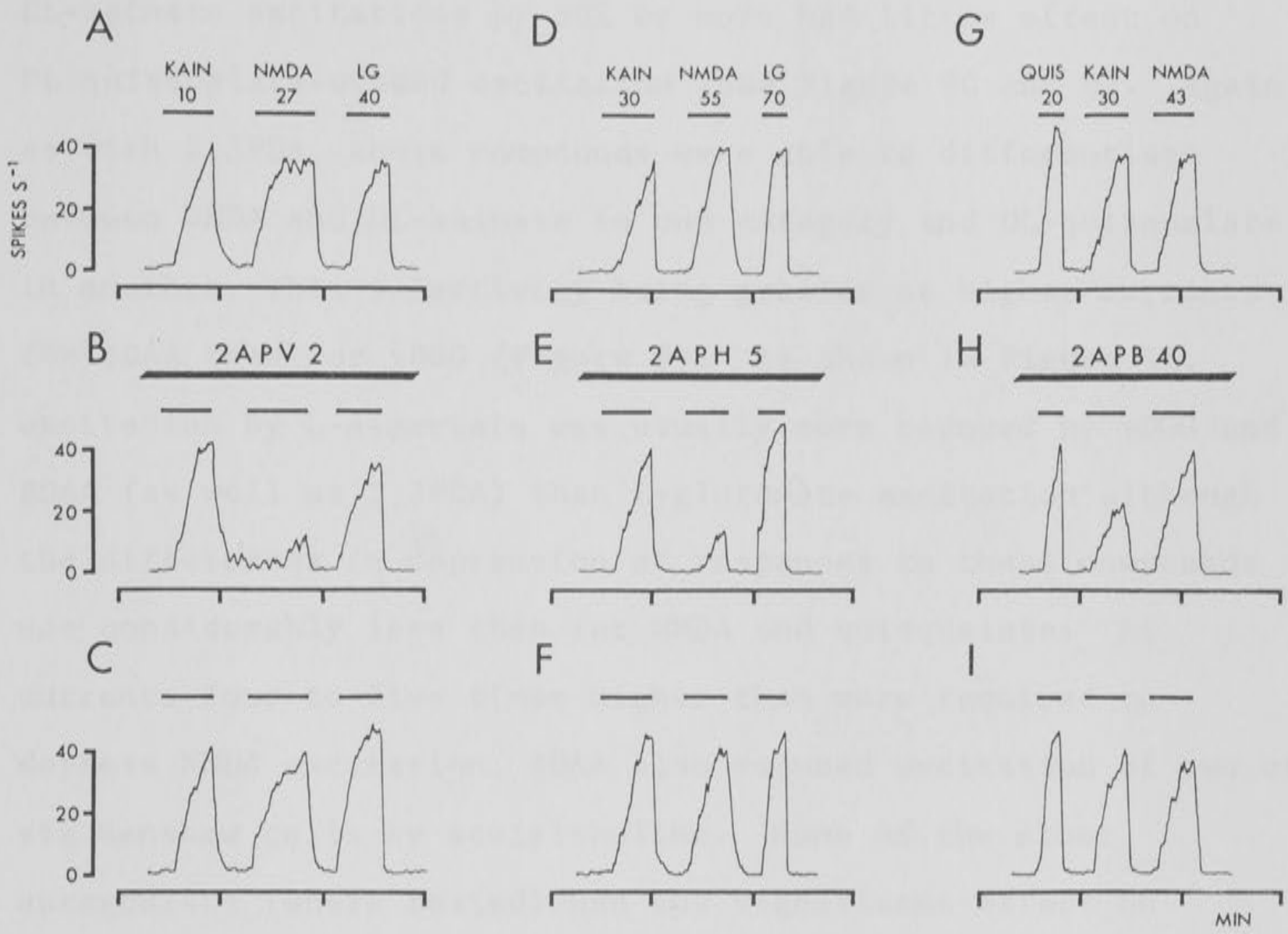


FIGURE 8 Effects of 2APV (A-C), 2APH (D-F) and 2APB (G-I) on the firing of three interneurones by KAIN, NMDA, L-glutamate (LG) and QUIS. A: before; B: during 2APV (2 nA); C: 15 min after a current of 80 nA was used to eject 2APV, totally suppressing excitation by NMDA but with no reduction of the effects of KAIN or LG; D: before; E: during 2APH (5 nA); F: 12 min after a current of 40 nA was used to eject 2APH, totally suppressing excitation by NMDA but with no effect on excitation by either KAIN or LG; G: before; H: during 2APB (40 nA); I: 3 min after 2APB.

antagonism of the excitation by DL-quisqualate and in general currents of γDGG or βDAA capable of reducing NMDA and DL-kainate excitations by 50% or more had little effect on DL-quisqualate-evoked excitation (see Figure 9C and D). Again, as with 2,3PDA, these compounds were able to differentiate between NMDA and DL-kainate in one category and DL-quisqualate in another, this selectivity being greater at higher currents for βDAA than for γDGG (Figure 9). As shown in Figure 11, excitation by L-aspartate was usually more reduced by γDGG and βDAA (as well as 2,3PDA) than L-glutamate excitation although the differences in depression of responses to these compounds was considerably less than for NMDA and quisqualate. At currents four to five times higher than were required to depress NMDA excitation, βDAA also reduced excitation of two of six Renshaw cells by acetylcholine. None of the other antagonists (where tested) had any significant effect on acetylcholine excitation. The effects of both γDGG and βDAA were also rapidly reversible.

(c) <u>The effects of phosphonate analogues on amino acid-induced excitation</u>.

2APV and 2APH were both very effective and reversible antagonists of NMDA, 2APV being slightly more potent than 2APH (Figure 10). Although recovery from 2APV was only slightly slower than that from 2,3PDA, the effect of 2APH was prolonged, full recovery often taking 5 min. Excitation by L-aspartate was also reduced by both 2APV and 2APH, being more depressed than DL-kainate-, DL-quisqualate- and L-glutamate-evoked excitation (Figure 11). Currents as low as 2 nA of either 2APV or 2APH

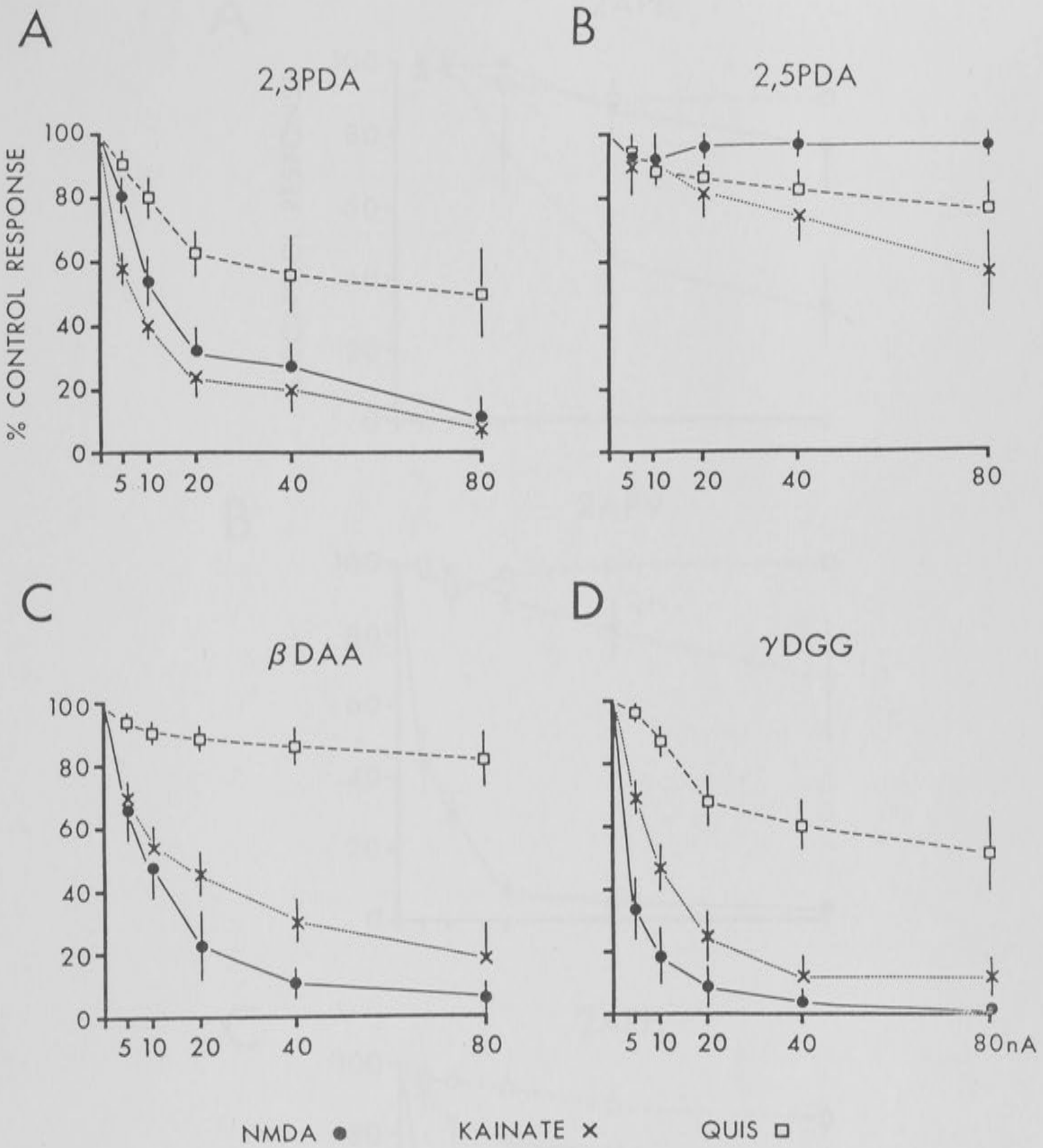


FIGURE 9 The effect of increasing concentrations (currents) of antagonists on the responses of spinal neurones to DL-quisqualate, DL-kainate and NMDA. In this Figure and Figures 10 and 11 each point represents the mean and standard error, and the number of cells for each agonist against each antagonist are shown in Table 1. Ordinates: Percent control response. Abscissae: current (nA).

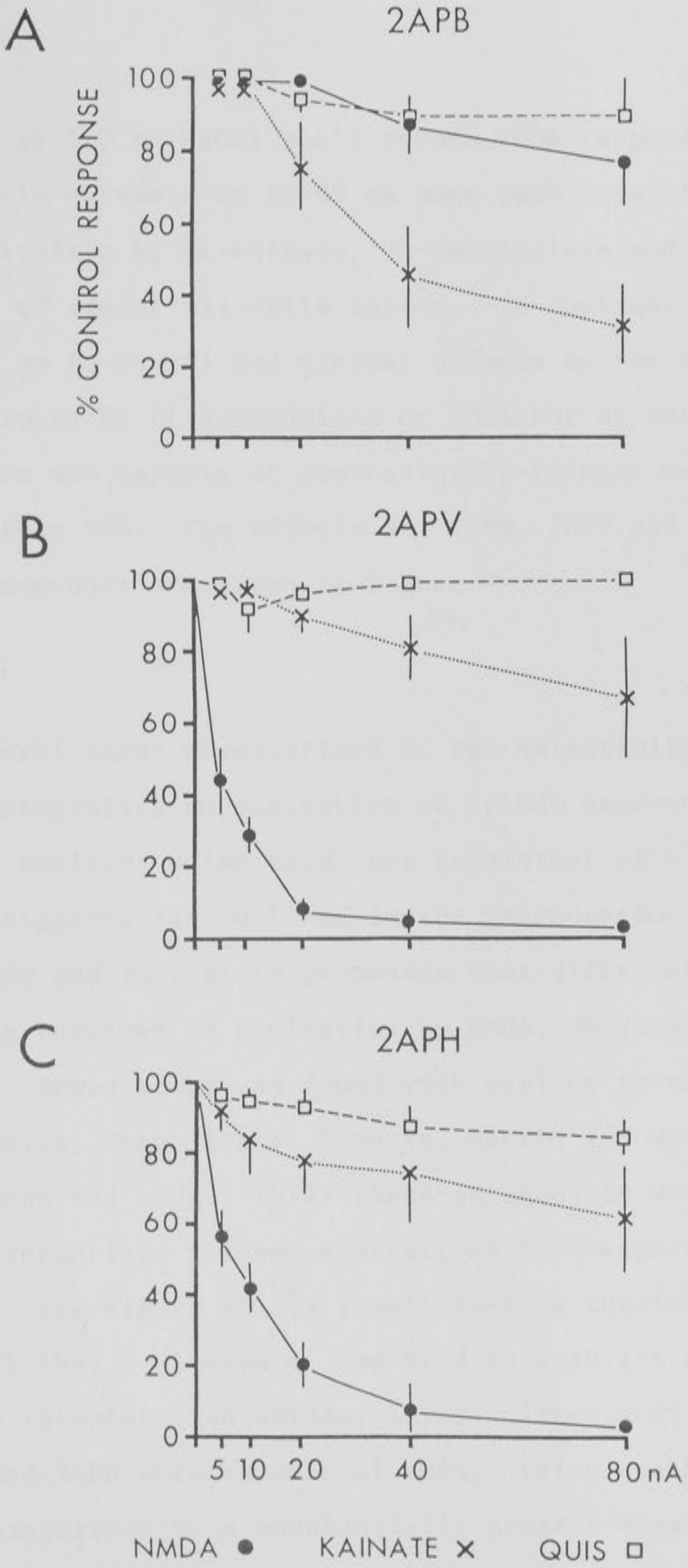


FIGURE 10 The effect of increasing concentrations (currents) of antagonists on the responses of spinal neurones to DL-quisqualate, DL-kainate and NMDA.

(both 50 mM in 150 mM NaCl) could reduce NMDA responses by more than 50% while currents up to 80 nA were much less effective against excitation by DL-kainate, DL-quisqualate and L-glutamate of almost all cells tested. In contrast 2APB (even at currents up to 80 nA) had minimal effects on the excitation of interneurones by DL-quisqualate or NMDA but at currents of 40 nA or more was capable of depressing DL-kainate excitations by greater than 50%. The effects of 2APB, 2APV and 2APH, on individual neurones are shown in Figure 8.

DISCUSSION

In general these observations of the selectivity of a number of antagonists on excitation of spinal neurones in the cat by some excitant amino acids are consistent with those of other investigators (as outlined in the Introduction to this chapter), and add support to proposals that different receptors are probably involved in excitation by NMDA, DL-quisqualate and DL-kainate. However, as was found with earlier investigations (Biscoe, Davies, Dray, Evans, Francis, Martin and Watkins, 1978; McLennan and Lodge, 1979) these antagonists were less able to differentiate between excitations by L-aspartate and L-glutamate (see Figure 11), a result that is consistent with the proposal that L-glutamate may bind to both its own and the L-aspartate receptor (Van Gelder, 1971). Irrespective of this, both 2APV and 2APH were capable of antagonizing excitation by NMDA and L-aspartate to a substantially greater degree than those by DL-kainate, DL-quisqualate and L-glutamate, and provides evidence for differences between the NMDA class of

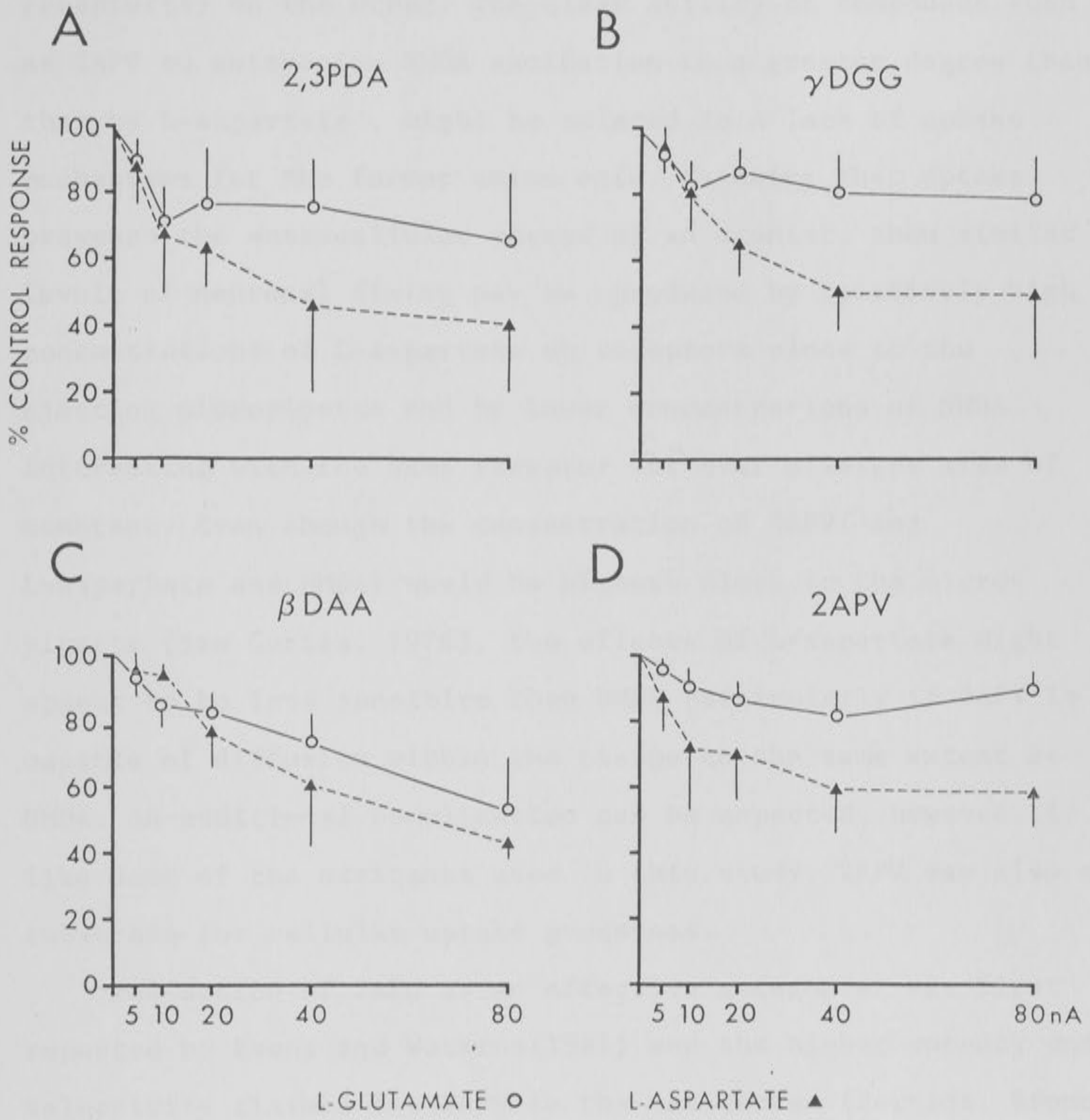


FIGURE 11 The effect of increasing concentrations (currents) of antagonists on the responses of spinal neurones to L-glutamate and L-aspartate.

receptor on the one hand and the kainate and quisqualate receptor(s) on the other. The clear ability of compounds such as 2APV to antagonize NMDA excitation to a greater degree than that by L-aspartate , might be related to a lack of uptake mechanisms for the former amino acid. Assuming that uptake prevents the extracellular spread of an agonist, then similar levels of neuronal firing may be produced by relatively high concentrations of L-aspartate at receptors close to the ejecting micropipette and by lower concentrations of NMDA interacting with the same receptor but over a larger area of membrane. Even though the concentration of 2APV(and L-aspartate and NMDA) would be highest close to the micropipette (see Curtis, 1976), the effects of L-aspartate might appear to be less sensitive than NMDA particularly if 2APV is capable of diffusion within the tissue to the same extent as NMDA. An additional complication can be expected, however, if, like some of the excitants used in this study, 2APV was also a substrate for cellular uptake processes.

The action of 2APH as an effective antagonist was first reported by Evans and Watkins(1981) and the higher potency and selectivity claimed for 2APH in the rat cortex (Perkins, Stone, Collins and Currry, 1981) does not seem to apply to cat spinal neurones. The present results resemble those of Evans, Francis, Jones, Smith and Watkins (1982) in the frog and rat spinal cords in vitro with little or no difference in selectivity between 2APV and 2APH, the former being slightly more potent and the latter longer acting. In contrast to these results, 2APB was ineffective against responses to L-glutamate, L-aspartate, NMDA and DL-quisqualate, selectively antagonizing

excitation by DL-kainate. In a previous study in the cat spinal cord (Watkins, Curtis and Brand, 1977) 2APB was ineffective against responses to L-glutamate, L-aspartate and DL-homocysteate although its effect on kainate excitation was not investigated. The results of Davies and Watkins (1982) have demonstrated different effects for the isomers of 2APB. Nevertheless, the use of 2APB to establish the presumed glutamergic nature of transmitters in the hippocampus in vitro (Cotman and Hamberger, 1978; White, Nadler, Hamberger, Cotman and Cummins, 1977) should be viewed cautiously. In this preparation, the use of 2APB appears to have been justified on the grounds of its reported effect as a postsynaptic L-glutamate antagonist at the locust neuromuscular junction (Cull-Candy, Donellan, James and Hunt, 1976) rather than on any demonstrated antagonism of the effects of various amino acid excitants on mammalian hippocampal neurones.

The two dipeptides γDGG and βDAA, had similar actions in line with their similar structures which differ only in the position of the peptide link (Watkins, 1981). These compounds were able to distinguish between NMDA and DL-kainate excitation on the one hand and DL-quisqualate excitation on the other. This selectivity appeared greater for βDAA. Similar results were obtained with 2,3PDA (see also Davies, Evans, Francis, Jones and Watkins, 1980) although this compound appeared marginally more effective against DL-kainate. 2,5PDA was only minimally effective against DL-kainate excitation and the selective glutamate antagonist action reported for this compound (Liu and McLennan, 1981) was not observed in this present investigation.

Such a difference in the antagonistic properties of 2,3PDA and 2,5PDA might have been expected from an examination of their structures (Figure 5) which suggests that 2,5PDA might not be as capable as 2,3PDA of attaining the correct conformational fit for interaction at central amino acid receptors.

Despite the lack of specific antagonists, this study has suggested that separate receptors do exist for quisqualate, NMDA and kainate. The selective antagonistic action of 2APV and 2APH has provided evidence for differences between the NMDA type receptor and those for kainate and quisqualate, and γDGG, βDAA and 2,3PDA have identified differences between receptors for NMDA and kainate and those for quisqualate. A similar grouping of amino acids, at least superficially, is apparent from the studies of Lambert, Flatman and Engberg (1981) showing differences in the underlying conductance mechanism in response to particular amino acids. Doubt concerning a common receptor for quisqualate and kainate has been previously expressed on pharmacological grounds (Hicks, Hall and McLennan, 1978; McLennan and Lodge, 1979) and on differences in conductance mechanisms recorded intracellularly from foetal mouse spinal cord neurones grown in vitro (MacDonald and Wojkowicz, 1980, 1982).

Although GDEE was not employed in this study it was used successfully in the investigations described in the previous chapter to selectively antagonize responses to DL-quisqualate, D- and L-AMPA and related compounds as compared to those by NMDA. Despite difficulty in differentiating between depression of L-glutamate and L-aspartate excitations (a problem encountered in the present study with other antagonists) and its

reduction of acetylcholine-evoked excitation of Renshaw cells, GDEE appears to be more selective between responses produced by NMDA and those by conformationally restricted compounds such as quisqualate and AMPA (but not ibotenate or kainate) and in this respect might prove to be useful in future studies designed to determine the conformational characteristics of central glutamate receptors.

(5) AMINO ACIDS IN SYNAPTIC TRANSMISSION: USE OF ANTAGONISTS

The high degree of selectivity of some of the recently synthesized antagonists against microelectrophoretically-ejected NMDA (as described in Chapter 4) suggested that they might be useful in determining a rôle for L-aspartate in central nervous transmission. Also 2APV has previously been used to implicate NMDA type receptors in mediating visual excitation of "sustained" cells in the cat retina (Ikeda and Sheardown, 1981, 1982), and polysynaptic excitation of interneurones and Renshaw cells in the cat spinal cord (Davies, Francis, Jones and Watkins, 1980) and the isolated spinal cords of the frog and immature rat (Evans, Francis, Jones, Smith and Watkins, 1982).

Although the dipeptide γDGG did not exhibit the selectivity for NMDA excitation seen with either 2APV or 2APH it has also been shown to be an effective antagonist of synaptic excitations. For example, γDGG (Francis, Jones and Watkins, 1980) decreased the synaptic depolarization of motoneurones in the isolated hemisected spinal cord of the frog, and selectively depressed dorsal root-, but not ventral root-evoked excitations of Renshaw cells in the cat (Davies and Watkins, 1981b; Davies, Francis, Jones and Watkins, 1980) and the low threshold sensory responses of rat caudal trigeminal nucleus neurones (Hill, Salt and Watkins, 1981). In the rat hippocampal slice preparation (Collingridge, Kehl and McLennan, 1981) Schaffer collateral input to CA1 pyramidal cells was reduced by γDGG as was the medial perforant path-evoked epsp in granule cells in the middle molecular layer of the dentate gyrus (Collingridge, Crunelli, Forda and Kelly, 1982). This

latter effect appeared to be presynaptic since it was not associated with a change in membrane potential, postsynaptic excitability or input resistance.

Perhaps because of its broad spectrum of antagonism against microelectrophoretically-ejected amino acids, 2,3PDA has multiple actions on spinal cord transmission. Davies, Francis, Jones and Watkins (1981) reported that 2,3PDA was capable of antagonizing the 2APV-sensitive dorsal root evoked (presumed polysynaptic) excitation of Renshaw cells in the cat spinal cord *in vivo*, and frog and immature rat spinal cord *in vitro*. At the same time this compound potentiated both the acetylcholine- and ventral root-evoked excitation of Renshaw cells. Also, 2,3PDA depresses both mono- and polysynaptic excitation of interneurones in the cat spinal cord (Davies and Watkins,1981a), the fast (presumed monosynaptic) component of the DR-VRP in the L_5 segment of isolated spinal cord preparations from immature rats (Evans, Smith and Watkins, 1981) and antagonized the excitation of rat caudal trigeminal neurones in response to low-threshold sensory stimulation (Hill, Salt and Watkins, 1980).

Complex patterns of antagonism have also been demonstrated for 2APB which microelectrophoretically has a similar, but weaker, capability of depressing responses to DL-kainate, DL-quisqualate and NMDA as 2,3PDA (Evans and Watkins, 1981). In the cat spinal cord(Davies and Watkins,1982), both isomers of 2APB depressed dorsal root evoked excitation in a number of dorsal horn interneurones and Renshaw cells so tested. Both monosynaptic and polysynaptic excitations were susceptible with

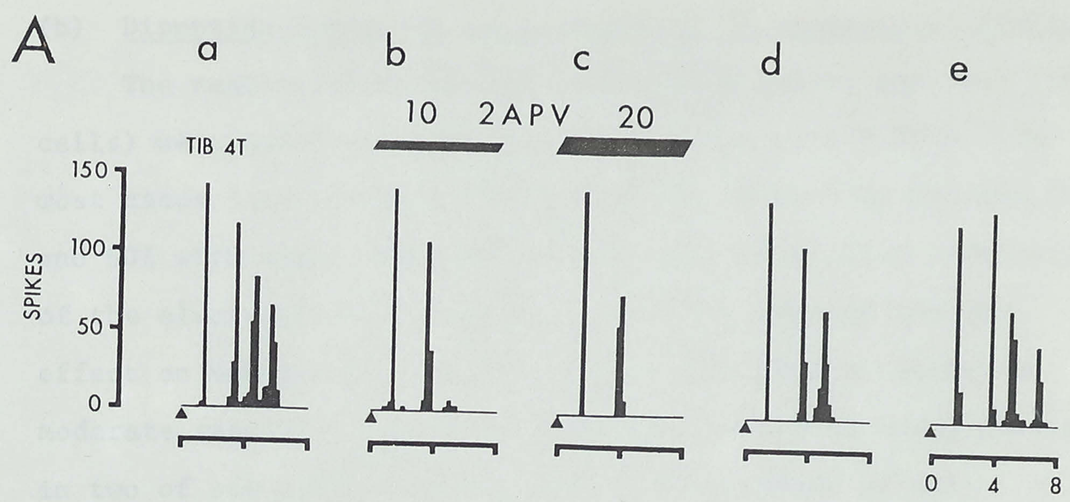
the L-isomer being the more potent at producing these effects. A possible presynaptic mode of action for the L-isomer is suggested by the results of Evans, Francis, Jones, Smith and Watkins (1982) who found in the isolated rat spinal cord preparation that the L-isomer was again more potent than the D-isomer in depressing the fast component of the DR-VRP but (as has also been reported by Davies and Watkins(1982) did not antagonize responses to amino acid excitants. In contrast, however, results from other studies (White, Nadler, Hamberger, Cotman and Cummins, 1977; Koerner and Cotman, 1981) showed that the perforant path input to dentate granule cells that originated in the lateral entorhinal cortex displayed high selectivity for inhibition by the L-isomer of 2APB but that this inhibition did not affect presynaptic fibre potentials or alter the quantity of glutamate released by elevated K^+. No information has been published on the actions of 2,5PDA, 2APH or βDAA on synaptic excitation in the cat. The effects of these compounds along with those previously reported (2APB, 2APV, γDGG and 2,3PDA) were investigated on the synaptic activation of identified interneurones and Renshaw cells. However, the effects of these antagonists on the excitation of Renshaw cells evoked by dorsal root volleys was not investigated.

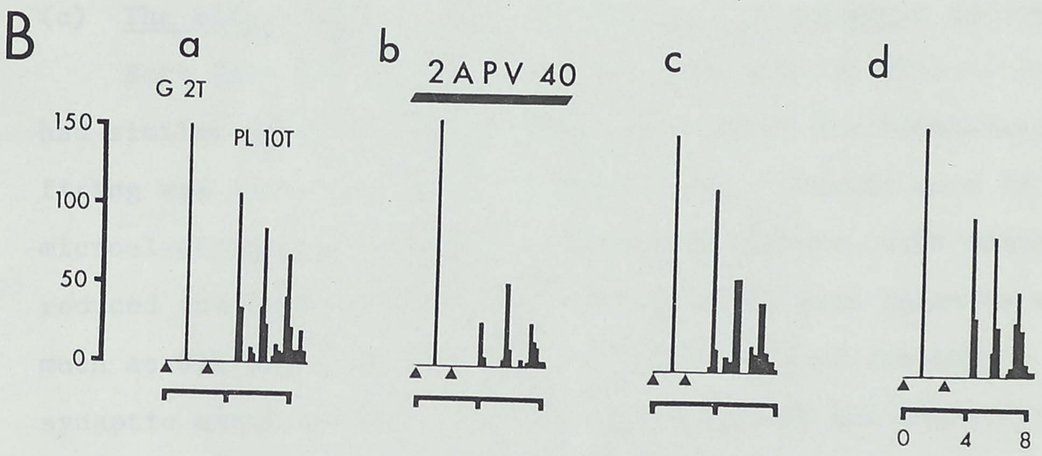
RESULTS

The effect of 2APV and 2,3PDA on synaptic excitation of individual neurones is shown in Figure 12 and summarized graphically for all cells in Figure 13. The number of cells tested with each antagonist is shown in Table 1 (Chapter 4) and the potency expressed relative to the most effective antagonist (2APV and 2APH; three arrows) of polysynaptic excitation.

(a) Effects of 2,3PDA and 2,5PDA on synaptic excitation

Figure 12C shows the effect of 2,3PDA (80 nA) on a non-spontaneously active interneurone excited monosynaptically and polysynaptically by stimulating the gastrocnemius nerve (2T). Polysynaptic firing was depressed reversibly by 70% whereas there was no significant change in either the latency or amount of monosynaptic firing (Figure 12Cb). Similar observations were made with six other dorsal horn interneurones which were not spontaneously active, using currents as high as 160-230 nA to eject 2,3PDA, which were sufficient to totally abolish excitation of these neurones by DL-quisqualate and NMDA. With four other interneurones, DL-quisqualate was used to maintain the background firing rate at a constant level (5-10 Hz) before, during and after 2,3PDA. DL-Quisqualate was selected for this purpose as the least affected excitant by 2,3PDA. These neurones were also excited monosynaptically by impulses in low threshold primary afferents, and responded polysynaptically to impulses in higher threshold fibres. As in Figure 12C, however, 2,3PDA ejected with currents as high as 80-100 nA, reduced polysynaptic excitation without any effect on that generated monosynaptically. The results for all cells are shown graphically in Figure 13. Recovery from the depression of synaptic excitation was rapidly reversible occurring within 1-2 min. In contrast to these results, 2,5PDA had no obvious effect on either the monosynaptic or polysynaptic firing of two interneurones and 2,3PDA did not alter the ventral root excitation of Renshaw cells (2,5PDA was not tested).





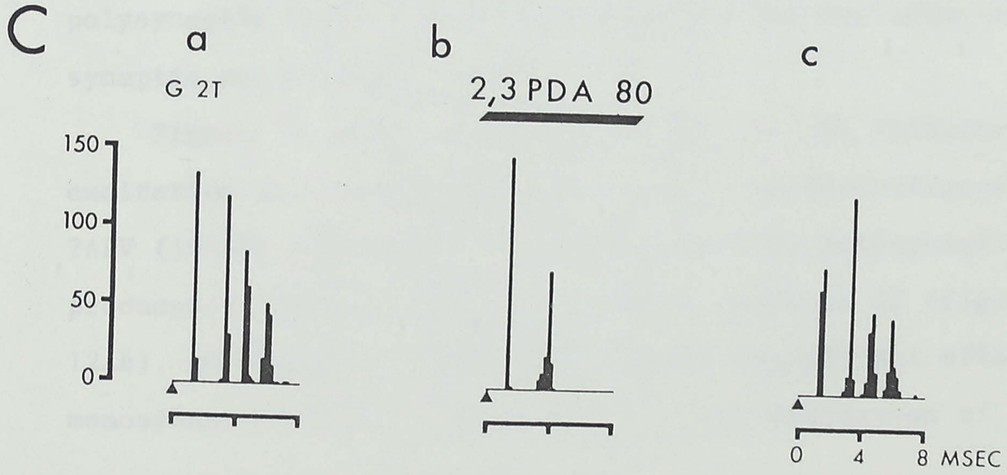


FIGURE 12 Peristimulus histograms of the effects of 2APV (A,B) and 2,3PDA (C) on the synaptic excitation of three dorsal horn interneurones. Within each series A and B background firing rates were maintained within 5% of the control level by the continuous ejection of DL-quisqualate (QUIS). Arrow heads beneath the histograms indicate the arrival of the fastest afferent volley at the cord, and the horizontal bars the electrophoretic administration of the antagonists. Abscissae: time, msec.

A: Interneurone excited mono- and polysynaptically by stimulation of the tibial nerve (TIB) at 4T; cell threshold 1.1T, monosynaptic latency 1.2 msec. a, before (QUIS - 28 nA); b, 3 min during 2APV (10 nA for a total of 7 min; QUIS - 35 nA); c, 4 min during 2APV (20 nA for 10 min; QUIS - 31 nA); d, 10 min after 2APV (QUIS -37 nA); e, 17 min after d (QUIS - 34 nA). Ordinate number of spikes on consecutive 0.2 msec bins for a total of 150 sweeps at 1 Hz.

B: Interneurone excited by stimulation of gastrocnemius nerve (G) at 2T (first arrow head), cell threshold 1.2T, central latency 1 msec, and the plantaris nerve (PL) at 10T (second arrow head), central latency 1.6 msec. a, before (QUIS - 60 nA); b, 3 min during 2 APV (40 nA for 10 min, QUIS - 60 nA); c, 6 min after 2APV (QUIS - 60 nA); d, 12 min after c (QUIS - 30 nA). Ordinate: as for A.

C: Interneurone excited by stimulation of the gastrocnemius nerve (G) at 2T, cell threshold 1.4T, central latency 1.2 msec. a, before; b, 5 min during 2,3PDA (80 nA for 6 min); c, 6 min after 2,3PDA. Ordinate: as for A.

(b) Dipeptide analogues as antagonists of synaptic excitation

The results obtained with γDGG (four cells) and βDAA (four cells) were qualitatively similar to those with 2,3PDA. In most cases late polysynaptic firing was reduced by between 50 and 60% with rapid recovery of the excitation after termination of the electrophoretic current. Neither compound had any effect on monosynaptic firing and despite βDAA producing a moderate reduction of Renshaw cell excitation by acetylcholine in two of six cells there was no obvious effect of this compound on ventral root excitation of Renshaw cells.

(c) The effect of phosphonate analogues on synaptic excitation

Both 2APV (15 of 16 cells) and 2APH (two of five cells) had similar effects. Under conditions where the background firing was maintained at an approximately constant rate by the microelectrophoretic ejection of DL-quisqualate, both compounds reduced the late polysynaptic excitation in some cases by as much as 85% while in only one cell 2APV reduced the monosynaptic excitation by 10%. Neither compound had any effect on the ventral root-evoked excitation of Renshaw cells. In comparison 2APB had only minimal and inconsistent effects on polysynaptic excitation (six cells) and did not alter monosynaptic excitation.

Figure 12 shows the effect of 2APV on the synaptic excitation of two interneurones. With the first(Figure 12A), 2APV (10 nA) selectively abolished the late polysynaptic firing produced by stimulation of the tibial nerve at 4T (Figure 12Ab), while even at 20 nA 2APV had no significant effect on monosynaptic firing (Figure 12Ac). Such depression of

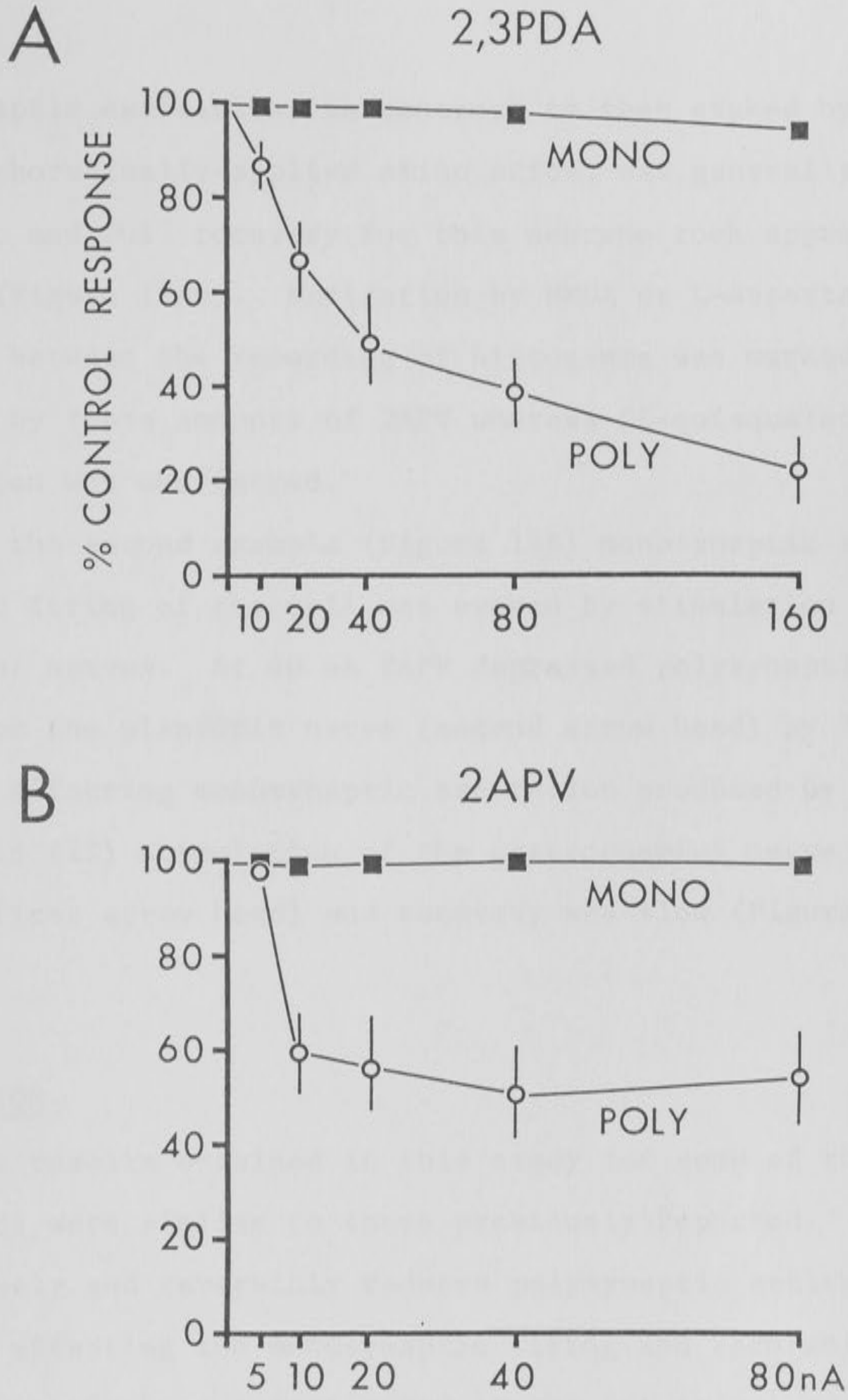


FIGURE 13 The effect of 2,3PDA and 2APV on the synaptic firing of spinal neurones. Depression of excitation was quantified by comparing the areas of histograms of synaptically-induced firing before and during ejection of the antagonist. Each point represents the mean and standard error. Ordinates: Percent control response. Abscissae: current (nA).

polysynaptic excitation, in contrast to that evoked by micro-electrophoretically-applied amino acids, was generally long lasting, and full recovery for this neurone took approximately 30 min (Figure 12Ae). Excitation by NMDA or L-aspartate ejected between the recording of histograms was markedly reduced by these amounts of 2APV whereas DL-quisqualate excitation was unaffected.

In the second example (Figure 12B) monosynaptic and polysynaptic firing of the cell was evoked by stimulation of two different nerves. At 40 nA 2APV depressed polysynaptic excitation from the plantaris nerve (second arrow head) by 70% without affecting monosynaptic excitation produced by low threshold (2T) stimulation of the gastrocnemius nerve (Figure 12Bb - first arrow head) and recovery was slow (Figure 12Bc and d).

DISCUSSION

The results obtained in this study for some of the compounds were similar to those previously reported. Thus 2APV selectively and reversibly reduced polysynaptic excitation without affecting the monosynaptic firing and 2APH which has not previously been investigated in the cat spinal cord, gave very similar results.

γDGG, βDAA and 2,3PDA also selectively but less potently reduced polysynaptic firing. No significant reduction of monosynaptic excitation was observed with 2,3PDA, however, a result which contrasts with the findings of Davies and Watkins (1981a). It is possible that differences in the action of 2,3PDA

may have resulted from the investigation of different populations of monosynaptically activated neurones and the use of DL-quisqualate in the present study to maintain neuronal excitability. Although this procedure was considered essential for the detection of any selective interference with a particular type of synaptic excitation (Curtis, Lodge, Bornstein and Peet, 1981), 2,3PDA did not influence monosynaptic excitation of neurones even in the absence of maintained background firing. 2,5PDA, which has been reported to selectively antagonize L-glutamate excitation (Liu and McLennan, 1981) was also ineffective against monosynaptic excitation.

In the electrophoretic studies (discussed in the previous chapter) 2APB produced moderate and selective antagonism of DL-kainate excitation, but was found to be without effect on either monosynaptic or polysynaptic excitation. Interestingly, 2APB has previously been shown to block the response of dorsal root fibres to DL-kainate and L-glutamate, DL-kainate having been shown to potently depolarize fibres in dorsal roots of immature rats (Davies, Evans, Francis and Watkins, 1979). The involvement, however, of kainate receptors in synaptic transmission still requires determination.

Undoubtedly, some of the complex observations made with 2APB resulted from use of the racemic mixture and more useful results might have been obtained in this study (and in Chapter 4) by use of the stereoisomers. Nevertheless, no evidence was found to justify the use of 2APB in establishing that glutamate is the transmitter of the perforant path input to the dentate

gyrus of the hippocampus (Koerner and Cotman, 1981; White, Nadler and Cotman, 1979) particularly since there is also conflicting biochemical evidence regarding the nature of the transmitter substance (Di Lauro, Schmid and Meek, 1981; Nadler and Smith, 1981).

This study has again demonstrated that it is possible pharmacologically to differentiate between monosynaptic and polysynaptic excitation of identified interneurones. This separation has also been shown previously with the D-isomers of the monoamino- and diaminodicarboxylates and the GABA analogue (-)-β-chlorophenyl-GABA (Curtis, Lodge, Bornstein and Peet, 1981) which selectively, and presynaptically, reduced monosynaptic excitation of interneurones in the intact spinal cord.

It is worth noting that the compounds capable of depressing polysynaptic excitation selectively reduced the longer latency firing. None of these antagonists showed blockade of the shorter latency (using the criteria outlined in Methods) polysynaptic firing. A possible explanation could be that this excitation is monosynaptic and results from impulses in slower conducting primary afferent fibres.

Thus although different transmitters may be released by different types of low threshold muscle or cutaneous primary afferent fibre (for example L-glutamate and substance P) these transmitters appear to be different from that released by a population of interneurones activated by impulses in higher threshold afferents whose transmitter is apparently capable of activating NMDA type receptors.

(6) POSTEXCITATORY DEPRESSION OF NEURONAL FIRING BY ACIDIC AMINO ACIDS AND ACETYLCHOLINE

During the initial investigations into the effect of microelectrophoretically-administered compounds on the firing rate of central neurones (Curtis, Phillis and Watkins, 1959, 1960; Curtis and Watkins, 1960, 1963) and Renshaw cells (Curtis and Eccles, 1958; Curtis, Phillis and Watkins, 1961) excitation of some cells was observed to be followed by a depression of the underlying spontaneous or background firing, a depression that appeared to be related to the preceding rate of excitation.

More recently McLennan and Liu (1981) published data on the postexcitatory effects of acidic amino acids on spinal interneurones and Renshaw cells of the urethane-anaesthetized rat. On cells that were either firing spontaneously or whose firing rate was maintained by the constant ejection of an excitant amino acid they made the following observations. Firstly, excitation of interneurones by L-glutamate or L-aspartate (but not D-aspartate, or ACh on Renshaw cells) was followed by a period of depressed background firing of some 20-200 sec duration. Secondly, Mclennan and Liu showed that these periods of postexcitatory depression (PED) following L-glutamate or L-aspartate could be blocked by the GABA antagonist bicuculline (Curtis, Duggan, Felix and Johnston, 1971) or the glycine (and ß-alanine) antagonist strychnine (Curtis, Hösli and Johnston, 1968).

On these grounds they proposed that extracellular decarboxylation of L-glutamate to GABA and L-aspartate to β-alanine could be the underlying mechanism of the PED which was not related to the preceding level of excitation.

Because of these conflicting results regarding the origin of PED it was considered important to reinvestigate this phenomenon.

RESULTS

(a) Amino acid and acetylcholine-evoked PED: effects of inhibitory amino acid antagonists

In the cell shown in Fiure 14, as with four other Renshaw cells, excitation by acetylcholine at low extracellular concentrations was followed by PED. With almost all interneurones investigated excitation by L-aspartate (37 of 38 cells), or L-glutamate (24 of 25 cells), was followed by a period of reduced background firing lasting (as with acetylcholine) from between 30 and 90 sec (see Figure 15A and C). In addition, PED occurred after excitation by D-glutamate (11 of 13 cells, Figure 17), D-aspartate (12 of 12 cells), DL-quisqualate (25 of 26 cells) but not after NMDA. In general, PED occurred whenever the maximum rate of firing exceeded 60-80 spikes s^{-1} for more than 10-15 s, although with some neurones much higher rates could be maintained for longer periods without evoking PED. PED also appeared to be more prominant when the rate of firing faded during the continued administration of excitants (see Curtis and Ryall, 1966b). The magnitude and duration of the reduced firing varied

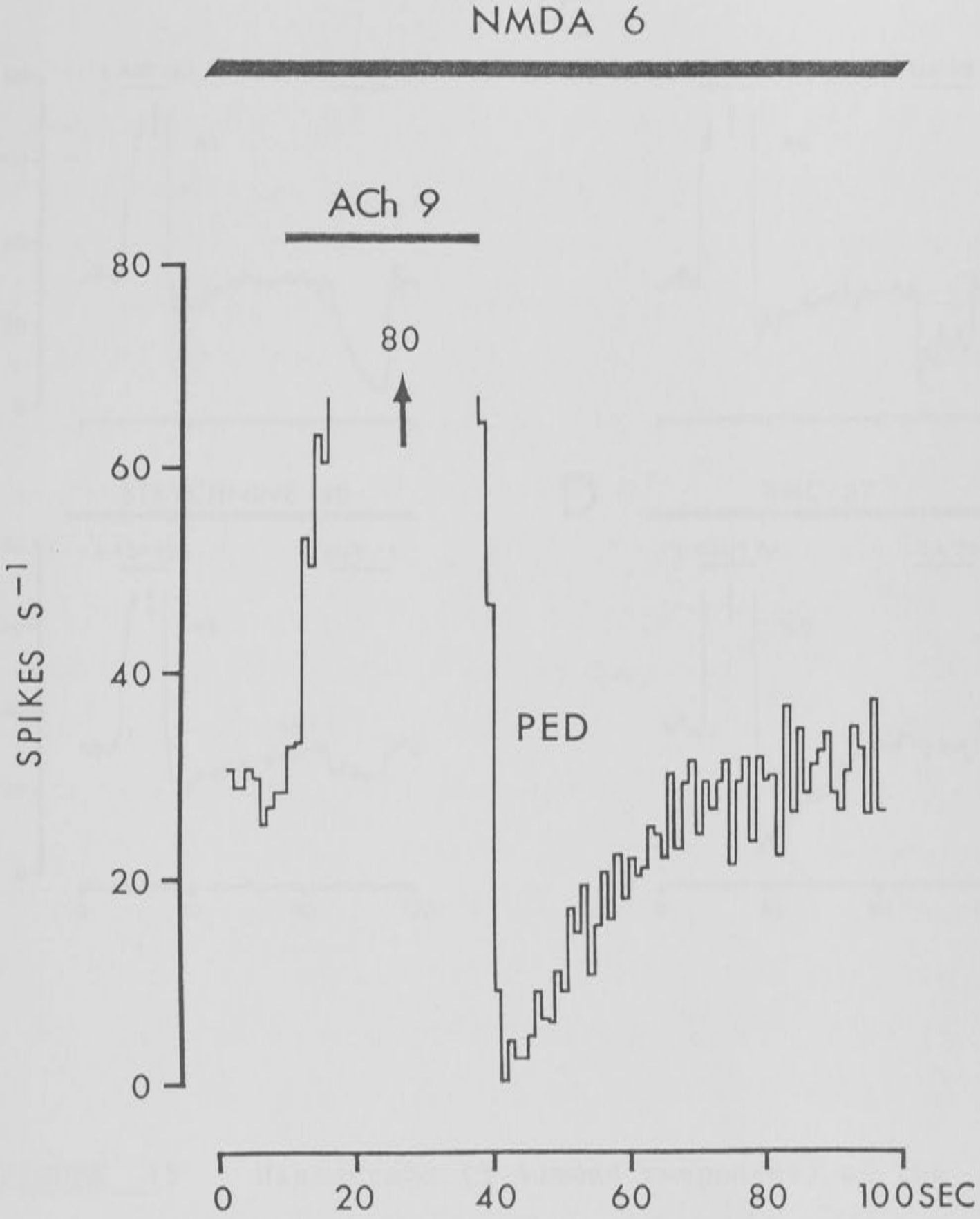


FIGURE 14 Histogram of the firing of a Renshaw cell in response to acetylcholine (9 nA). Background firing was maintained by the continuous ejection of NMDA (6 nA). In this and all other Figures in this Chapter electrophoretic ejections are indicated by horizontal bars. The figure adjacent to the arrows within the truncated records give the peak firing rate. Ordinates: firing frequency, spikes s^{-1}. Abscissae: time, sec.

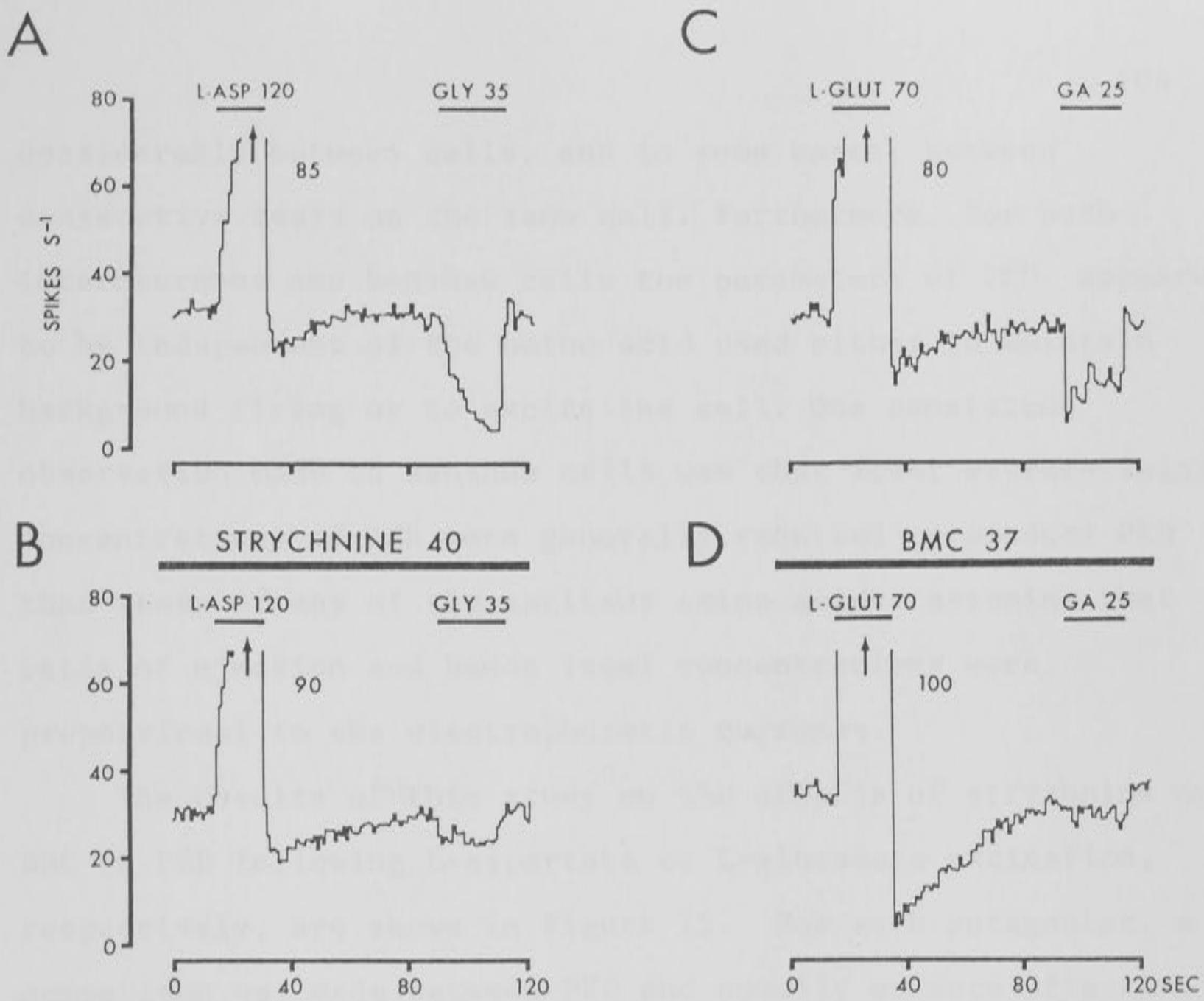


FIGURE 15 Histograms (3 summed responses) of the firing of a spinal dorsal horn interneurone, maintained by quisqualate and excited by L-aspartate (L-ASP 120 nA), L-glutamate (L-GLUT 70 nA) and inhibited by glycine (GLY 35 nA), GABA (GA 25 nA). A, before and B, during strychnine (40 nA for 8 mins); C, before and D, during BMC (37 nA for 8 mins). Ordinates and Abscissae: as for Figure 14.

considerably between cells, and in some cases, between consecutive tests on the same cell. Furthermore, for both interneurones and Renshaw cells the parameters of PED appeared to be independent of the amino acid used either to maintain background firing or to excite the cell. One consistent observation made on Renshaw cells was that lower extracellular concentrations of ACh were generally required to produce PED than those of any of the excitant amino acids, assuming that rates of ejection and hence local concentrations were proportional to the electrophoretic currents.

The results of this study on the effects of strychnine and BMC on PED following L-aspartate or L-glutamate excitation, respectively, are shown in Figure 15. For each antagonist, a comparison was made between PED and equally or more effective concentrations of an appropriate inhibitory amino acid; in the case of L-aspartate, glycine was used since the inhibitory effects of glycine and β-alanine are similarly sensitive to strychnine (Curtis, Hösli and Johnston, 1968). Even when sufficient strychnine was ejected to block the effect of glycine on eight cells, the excitatory effect of L-aspartate was not significantly enhanced, as might have been expected if this excitant was being continuously converted to β-alanine, and the duration or the extent of PED was unaffected. This is illustrated for a cell in Figure 15A and B. With BMC, it was usual to find an increased PED under conditions where the inhibitory action of GABA was reduced (Figure 15C and D). This might be related to the increased firing rate due to the reported effects of BMC in altering cell excitability (Curtis, Duggan, Felix, Johnston and Mclennan,1971).

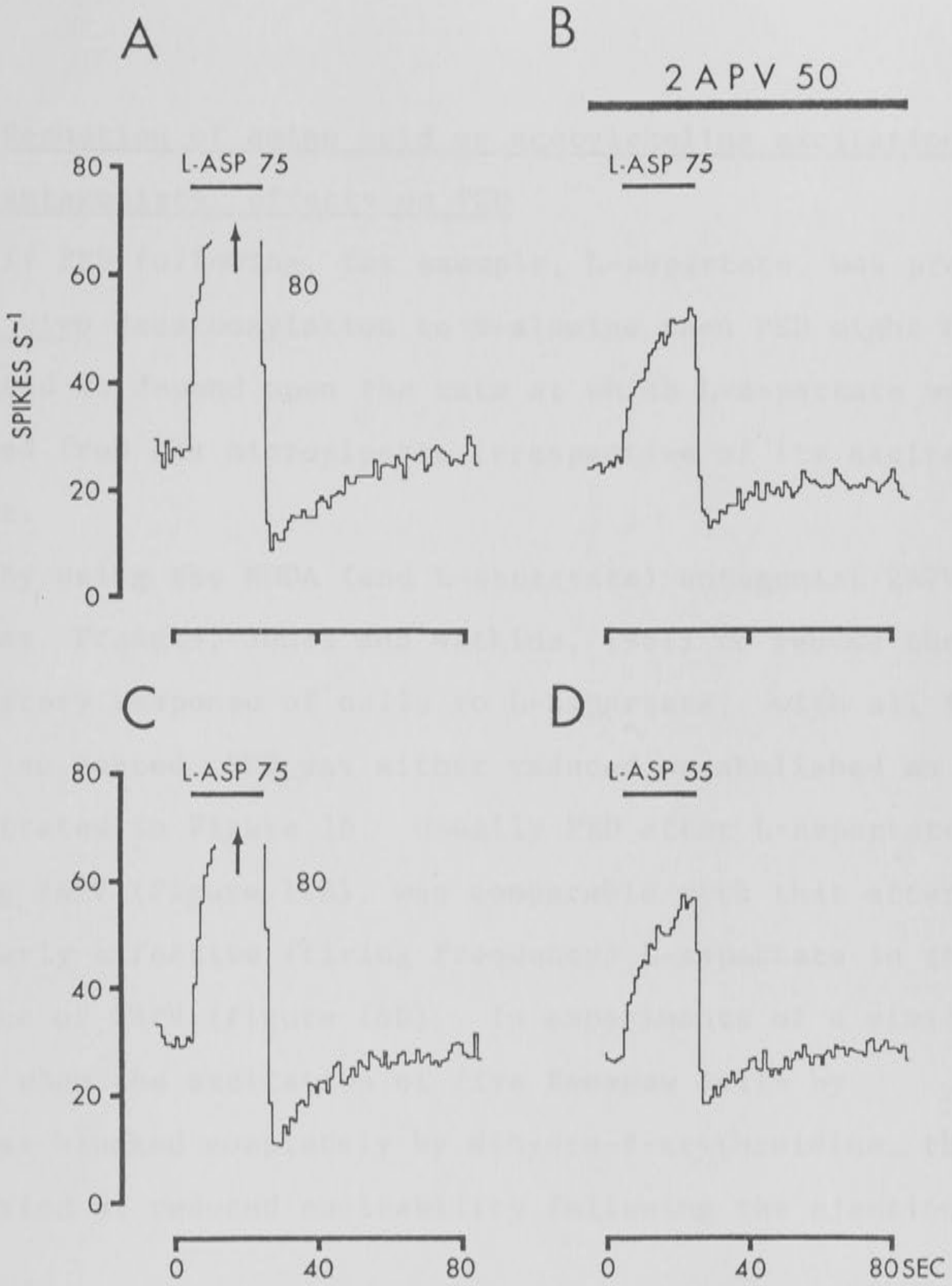


FIGURE 16 Histograms of the firing of a spinal dorsal horn interneurone maintained by DL-quisqualate and excited by L-aspartate (75 nA). A, before; B, during 2APV (50 nA for 5 mins); and C, ;7 mins after 2APV. D. firing of the same interneurone by L-aspartate (55 nA) to produce a maximum firing rate similar to that in B. Ordinates and Abscissae: as for Figure 14.

(b) Reduction of amino acid or acetylcholine excitations by antagonists: effects on PED

If PED following, for example, L-aspartate, was produced by *in vivo* decarboxylation to B-alanine then PED might be expected to depend upon the rate at which L-aspartate was ejected from the micropipette irrespective of its excitatory effect.

By using the NMDA (and L-aspartate) antagonist 2APV (Davies, Francis, Jones and Watkins, 1981) to reduce the excitatory response of cells to L-aspartate, with all five cells so tested, PED was either reduced or abolished as illustrated in Figure 16. Usually PED after L-aspartate, but during 2APV (Figure 16B), was comparable with that after similarly effective (firing frequency) L-aspartate in the absence of 2APV (Figure 16D). In experiments of a similar type, when the excitation of five Renshaw cells by ACh was blocked completely by dihydro-β-erythroidine, there was no period of reduced excitability following the ejection of ACh.

(c) Changes in the amount or duration for ejection of excitants: effect on PED.

In order to investigate such effects the response of a cell to either (A) increasing currents of an excitant for a fixed duration or (B) increasing duration at a fixed current was determined. In the example shown in Figure 17A, excitation by D-glutamate initially ejected at 110 nA was followed by no PED, but subsequently increasing the current for ejection increased the firing rate from approximately 50 to 80 spikes s^{-1} and PED was observed. In Figure 17B D-glutamate (210nA)

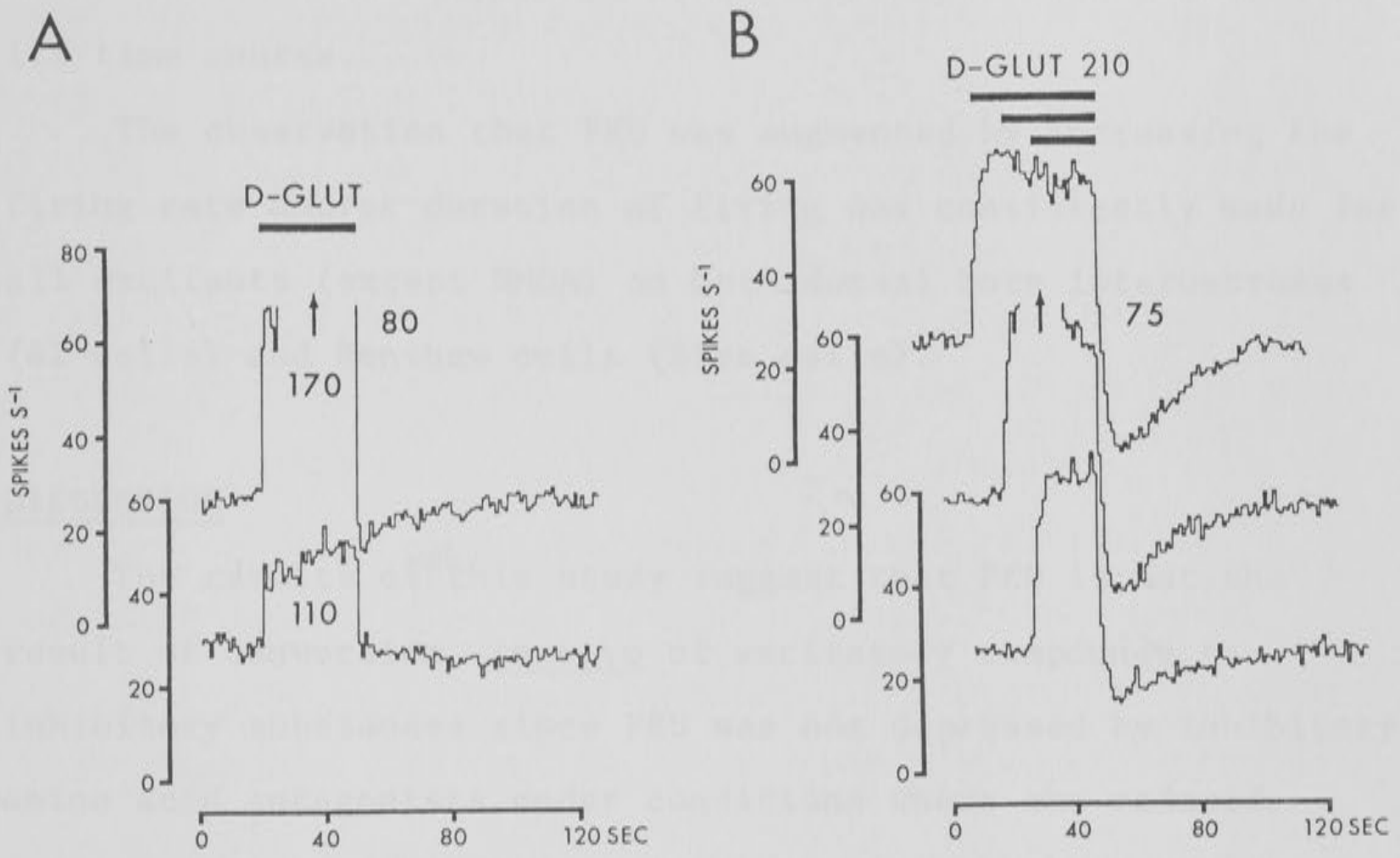


FIGURE 17 Histograms of the firing of a spinal dorsal horn interneurone maintained by NMDA. A, excitation by D-glutamate (D-GLUT, 110 and 170 nA) for 30 sec; B, by D-glutamate (210 nA) for 20, 30 and 40 sec. Abscissae: as in Figure 14, but displaced to the left for upper records. Ordinates: as for Figure 14.

was firstly ejected for 20 s producing a small but obvious PED. Increasing the duration (without significant increase in firing rate) enhanced the amplitude of the PED with minimum effect on its time course.

The observation that PED was augmented by increasing the firing rate and/or duration of firing was consistently made for all excitants (except NMDA) on both dorsal horn interneurones (41 cells) and Renshaw cells (five cells).

DISCUSSION

The results of this study suggest that PED is not the result of conversion *in vivo* of excitatory compounds to inhibitory substances since PED was not depressed by inhibitory amino acid antagonists under conditions where the reduced excitability by inhibitory amino acids was blocked. Furthermore, PED was observed after ACh, D-glutamate, D-aspartate and DL-quisqualate as well as L-glutamate and L-aspartate. Although it is conceivable that quisqualate could be decarboxylated *in vivo* to the inhibitory compound quisqualamine (Evans, Francis, Hunt, Martin and Watkins, 1978) it is unlikely that ACh could be converted into any potent inhibitory compound: choline, for example, neither excites nor depresses Renshaw cells (Curtis, Phillis and Watkins, 1961). In addition D-glutamate (Roberts and Frankel, 1951) and D-aspartate are presumably poor substrates for mammalian decarboxylases. Furthermore, enzymic conversion of L-glutamate and L-aspartate to relatively short acting inhibitors, such as GABA and β-alanine, is unlikely to account for the prolonged duration of PED.

PED could not, however, be demonstrated after NMDA. Recovery after excitation of neurones by this amino acid is slow compared with that by other excitant amino acids (Curtis and Watkins, 1963) presumably reflecting both inefficient uptake and slow dissociation from receptors. Such a slow recovery to baseline excitation may have masked a concomitant decrease in background firing. The present observations clearly differ from the biphasic responses to DL-ibotenate recorded on cat spinal interneurones. Excitation by this amino acid was followed by a period of reduced excitability which could last up to one hour (MacDonald and Nistri, 1977, 1978). Since this depression could be antagonized by BMC, it was suggested (Curtis, Lodge and McLennan, 1979) that ibotenate was decarboxylated in vivo to the potent GABA analogue muscimol. Although other mechanisms have been suggested to account for this effect (MacDonald and Barker, 1980; Nistri, 1981) it is obvious that the time course of that inhibition is considerably longer than that recorded in the present study.

In contrast to the results of McLennan and Liu (1981) this investigation has shown that the extent of PED depended upon the magnitude of the preceding excitation, and that PED could be increased by either increasing the duration or the current ejecting an excitant. It could be argued, however, that raising the current or time for ejection also increased the amount of amino acid ejected with subsequently more substrate available for decarboxylation. If this were the case then PED should have been observed irrespective of any preceding excitation, but reduction of the excitant action of L-aspartate by 2APV or of ACh by DHβE, resulted in a decrease or total loss of PED.

The involvement of *in vivo* decarboxylation of excitant amino acids in the generation of PED seems unlikely in the cat. The results suggest that PED is in fact a direct result of the preceding level of excitation. Thus PED is probably the consequence of an increased intracellular sodium concentration as a direct effect of excitant amino acid depolarization and generation of action potentials which activates an electrogenic sodium pump with subsequent membrane hyperpolarization. The activation of such a pump would also explain the fading of the level of excitation during the electrophoretic administration of excitants which has previously been reported (Curtis and Ryall, 1966b). Further stimuli for increased pump activity include the influx of sodium coupled with that of amino acids (Johnstone, 1979) or the accumulation of extracellular potassium as has been described for the sodium pump in canine Purkinje fibres (Kline and Kupersmith, 1982).

Support for a hyperpolarizing membrane pump comes from the studies of Ransom, Barker and Nelson (1975) who demonstrated by intracellular recording from cultured mouse spinal neurones that excitation by microelectrophoretic L-glutamate was followed by a long lasting (20-200 s) hyperpolarization which was related to the duration and amount of the L-glutamate current. This postglutamate hyperpolarization was not associated with a change in input resistance, did not have an apparent reversal potential and was found to be rapidly and reversibly abolished by the cardiac glycoside stropthanthidin, an inhibitor of the activity of $Na^{+}K^{+}$- ATPase. The existence of a partly electrogenic sodium pump was also proposed (Koike, Mano, Okada and Oshima, 1972) to account for

the hyperpolarizing effect of intracellularly injected sodium ions on the membrane potential of cat pyramidal tract cells. Also, the injection of sodium ions into these cells decreased the maximum rates of rise and fall of the spike potential. Alterations in the shape of extracellularly recorded action potentials, which are generally more apparent for excitant amino acids which are effective substrates for amino acid uptake processes (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972), and which may culminate in "depolarization block", are presumably associated with increases in membane conductance and depolarization and inactivation of localized regions of membrane (see Curtis, Phillis and Watkins, 1960). The influence of local and general metabolic factors on the activity of membrane pumps and the extent to which transmembrane ion concentrations can be perturbed before pump activation results, would be expected to determine the generation and duration of PED.

The generation of PED in this study and the associated phenomena of changes in the configuration of extracellularly recorded action potentials and fading excitatory responses, previously reported, may be the result of the non-physiological rates and duration of firing in response to high concentrations of excitants delivered by the microelectrophoretic technique. Differences in the relationship between PED and firing frequencies for different neurones might be expected from the different firing characteristics of various types of neurones under "normal" conditions. Observations made of hyper-polarization after post-tetanic stimulation of dorsal spinocerebellar tract neurones of the cat led Kuno, Miyahara and Weakly (1970) to propose a physiological rôle for PED in

impulse coding and self-biasing. Such a rôle may also be suggested for the strychnine-, bicuculline- and tetanus toxin-insensitive pause which follows high frequency synaptic excitation of Renshaw cells (Curtis, Game, Lodge and McCulloch, 1976), although it is most unlikely that under physiological conditions any one Renshaw cell is subjected to excitation by simultaneous impulses in virtually all fibres of the appropriate ventral root.

7. The effects of GABA, related amino acids and amines on the electrical threshold of ventral horn group Ia afferent terminations in the cat.

In previous studies in the mammal (Sverdlov and Kozhechkin, 1975; Gmelin and Cerletti, 1976; Curtis, Lodge and Brand, 1977) and amphibian (Barker, Nicoll and Padjen, 1975) in which the microelectrophoretic effect of GABA on central fibres of primary afferent neurones was investigated, this compound increased the excitability of these endings, such a result being interpreted as a depolarization of the terminations. In addition, GABA depolarized the cell bodies of these neurones in dorsal root ganglia(see Section c, of the General Introduction) and it has therefore been assumed that GABA receptors are distributed upon the membranes of the soma as well as the central terminals (Evans, 1980). Prolonged application of kainate or N-methyl-D-aspartate to frog hemicords in this latter study also depolarized (recorded in the dorsal roots) and although L-glutamate and other excitatory amino acids do not depolarize dorsal root ganglion cells (de Groat, 1972) they do depolarize the central terminals of primary afferent neurones in both mammals (Curtis, Lodge and Brand, 1977; Evans, 1978) and amphibian (Barker, Nicoll and Padjen, 1975). Although it may be envisaged that the terminations of primary afferent neurones also have receptors for excitant amino acids, this action may be indirect and related to an increase in extracellular K^+ in response to local excitation of neurones by amino acids (Evans, 1980). Elevation of K^+ in the extracellular medium had previously been proposed as a possible contributing factor

to the depolarization of primary afferent terminals by sensory volleys (Curtis, Duggan, Felix and Johnston, 1971, Bruggencate, Lux and Lieble, 1974, Krnjević and Morris, 1975). A recent investigation (Curtis and Lodge, 1982), however, reported that this depolarization by K^+ appeared not to involve changes in membrane conductance. This study employed the central barrel of a multibarrel micropipette as an extracellular stimulating microelectrode to determine changes in the electrical thresholds of single terminations of identified primary afferent fibres (see Methods), and compared the effects of microelectrophoretic GABA and other substances with the depolarization generated synaptically. The reduction in threshold evoked by GABA and that produced by tetanic stimulation of low threshold flexor afferents (PAD) were diminished by microelectrophoretic BMC. This GABA antagonist alone elevated the threshold of some terminations but had no effect on depolarization by either K^+ or L-glutamate (see also Davidoff, Hackman and Osorio, 1980). Furthermore, GABA increased terminal membrane conductance since threshold reductions by GABA, but not K^+ or L-glutamate, were associated with a decrease in PAD. Neither GABA nor BMC influenced the threshold or afferent depolarization of non-terminal regions of Ia fibres.

This latter finding of the investigations of Curtis and Lodge (1982) suggests that receptors associated with the depolarizing action of GABA are located on the central terminal portions of primary afferent fibres. Such a requirement for this type of experiment, therefore, is that the termination being stimulated is sufficiently close to the tip of the

micropipette such that it is influenced by pharmacologically active compounds administered from the other barrels of the micropipette (Sverdlov and Kozhechkin, 1975). In a previous study (Curtis, Lodge and Brand, 1977) it was reported that the sensitivity of terminations to GABA was related to the intensity of the stimulating current; the majority of terminals unaffected by GABA had thresholds exceeding 1 μA. Those with thresholds less than this value could be influenced by amino acids and other compounds ejected microelectrophoretically with currents of less than 100 nA (see also Sastry, 1979; Rudomin, Engberg, Jankowska and Jimenez, 1980).

In the study of Curtis and Lodge (1982) the depolarization of Ia terminations by GABA was found to be frequently transient, the initial bicuculline-sensitive effect diminishing and even being replaced by an increase in threshold. Such an increase was the only effect of GABA on some Ia terminations, and was the major effect observed in an earlier investigation (Curtis and Ryall, 1966a) in which no attempt was made to locate individual terminations, and excitability changes were related to the number of Ia afferent fibres activated by stimulation near motoneurones. Recently, Rudomin, Engberg, Jankowska and Jimenez (1980) and Rudomin, Engberg and Jimenez (1981) found that GABA reduced the electrical threshold of the terminal portion of gastrocnemius Ia afferents but the thresholds of nearby, vestibulo- and rubrospinal fibres were either increased or unchanged but never lowered by GABA applied in the same current ranges. This latter effect was interpreted as a hyperpolarization, the consequences of increases in membrane ionic conductance similar

to those by which GABA hyperpolarizes red nucleus neurones (Altmann, Steinberg, Bruggencate and Sonnhof, 1972). However, rubrospinal fibre terminals were found to be depolarized by stimulation of sensory neurones, such depolarization not being attributed to the activation of gabergic pathways (as for segmental Ia afferents) but possibly to the extracellular accumulation of K^+.

A series of other papers using excitability techniques have reported increases in threshold of single primary afferents in response to other microelectrophoretically-ejected compounds. Thus, in anaesthetized or spinal cats, substance P (Randić, 1981; Randić, Carstens, Zimmermann and Klumpp, 1982) applied electrophoretically, by pressure microinjection, or systemically, produced dose-related reversible decreases or increases in threshold for antidromic activation of both C- and A-fibres. In addition, two other papers have reported that in the cat dorsal horn, noradrenaline (NA; Jeftinija, Semba and Randić, 1981) and 5-hydroxytryptamine (5HT: Carstens, Klumpp, Randić and Zimmermann, 1981) compounds which depress cat dorsal horn neurones (Belcher, Ryall and Schaffner, 1978; Headley, Duggan and Griersmith, 1978), increased the threshold of cutaneous C fibres and the threshold of both A and C fibres, respectively.

This chapter reports the results of an investigation into the mechanism responsible for the increase in threshold observed with GABA, and provides evidence that this increase is related not to effects on membrane conductance but to alterations in the ionic concentration of the extracellular medium consequent upon the Na^+-dependent uptake of amino acids and amines.

RESULTS

The effects of a number of amino acids as well as NA and 5HT were compared with those of GABA on the electrical thresholds of extensor Ia terminations excited near lumbar motoneurones. The amino acids (see Table 2) were divided into three groups on the basis of their ability to inhibit in a bicuculline-sensitive fashion the firing of cat spinal interneurones and to inhibit the Na^+-dependent uptake of GABA by mammalian CNS tissue in vitro . The results for both NA and 5HT are not included in this Table but are described in the text. Each compound was tested on at least five terminations, and in at least two animals, the depolarizing action of amino acids having biphasic effects being compared using relatively low electrophoretic currents (5-40 nA) during which depolarizations were maintained at approximately plateau levels. Because of difficulties in measuring quantitative differences between the effects of amino acids, the biphasic GABA-like actions of some, and the limited number of comparisons which were made, potencies for depolarizing action (Table 2, column 6) were expressed in a similar fashion to that used for the inhibition of neurones(Table 2, column 2).

(a) Effects of amino acids on termination thresholds

Group A amino acids

The compounds included in this group are all structural analogues of GABA, their inhibitory action on neurones being more prolonged and potent than that of the parent compound. They are poor substrates for GABA uptake processes in vitro (Table 2, column 3) which might partly explain why, apart from

TABLE 2 COMPARATIVE EFFECTS OF AMINO ACIDS ON SPINAL NEURONES, Ia TERMINATIONS, COUPLING RESISTANCES AND IN VITRO PREPARATIONS.

	1	2	3	4	5	6	7
	AMINO ACID	INHIBITION OF NEURONES	IC_{50} FOR GA UPTAKE (μM)	UPTAKE	CHANGE IN TERMINATION THRESHOLD	POTENCY RELATIVE TO GABA	EFFECT ON COUPLING RESISTANCE
GROUP A	MUSCIMOL	+	>1000	D	↓	+	↑
	DIHYDROMUSCIMOL	+	>1000		↓	+	
	PIPERIDINE-4-SULPHONIC	+	>5000		↓	+	↑
	3-AMINO-PROPANE-SULPHONIC	+	>1000		↓	+	
	ISOGUVACINE	+	ns		↓	+	
	ISONIPECOTIC	=	>5000		↓	=	↑
	KOJIC AMINE	=	>100		↓	=	
	THIP	=	ns		↓	=	↑
GROUP B	GABA	=	20	D	↓↑	=	↑
	DL-3-HYDROXY-GABA	-	100	I	↓↑	-	
	DL-2-CHLORO-GABA	+	59	I	↓↑	+	
	trans-4-AMINO-CROTONIC	=	24	I	↓↑	=	
	4-METHYLAMINO-BUTYRIC	-	23	I	↓↑	-	
	4-AMINOVALERIC	-	9	I	↓↑	-	↑
	DL-2-HYDROXY-GABA	-	1	I	↓↑	=	
	IMIDAZOLE-4-ACETIC	=	>1000	see text	↓↑	=	↑
	D-NIPECOTIC		5	D	↓↑		
GROUP C	GLYCINE	0	>1000	D	↑	0	↑
	β-ALANINE	0	>1000	D	↑	0	↑
	TAURINE	0	>1000	D	↑	0	↑
	L-HISTIDINE		>1000	D	↑	0	↑
	L-2,4-DIAMINO-BUTYRIC		40	D	↑	0	↑
	2,2-DIMETHYL-β-ALANINE		ns	I	↑	0	
	DL-ORNITHINE	0		I	↑	0	
	L-LYSINE	0		D	↑	0	
	L-2,3-DIAMINO-PROPIONIC	0		I	↑	0	
	L-ARGININE	0		I	↑	0	↑
	1	2	3	4	5	6	7

TABLE 2 Comparison of the effects of GABA and a number of amino acids on the electrical thresholds of extensor Ia terminations excited near lumbar motoneurones. Column 2 indicates the potency of the amino acids to inhibit the firing of cat spinal interneurones; more than (+), equal to (=) or less than (-) that of GABA. The ability of these amino acids to inhibit the Na^+-independent uptake of GABA by mammalian CNS tissues in vitro is shown in column 3[1]. (μM concentration for 50% inhibition, IC_{50}). Since poor substrates for GABA transport systems, however, may be taken up into neurones and glia by other carriers, for example glycine (Iversen and Neal, 1968) column 4 indicates Na^+ dependent uptake of individual amino acids by mammalian brain preparations in vitro, measured directly (D), or indirectly (I) in cross-inhibition studies without reference to species and regional differences or to 'high' or "low" affinity systems (see Martin, 1976) as defined by kinetic data[2]. The duration of changes in threshold (column 5) are indicated by arrows; (↓) reduced threshold (depolarization), (↑) increased threshold, (↓↑) biphasic GABA-like effects. In column 6 the potencies for the depolarizing action of these amino acids are expressed in a similar fashion to that used for the inhibition of neurones and column 7 indicates increases in the extracellular coupling resistance.

1. Iversen and Neal, 1968; Iversen and Johnston, 1971; Beart and Johnston, 1973; Brehm, Krogsgaard-Larsen, and Jacobsen, 1979; Johnston, Allan, Kennedy and Twitchin, 1979).
2. Cohen and Lajtha, 1972; Brehm, Krogsgaard-Larsen and Jacobsen, 1979; Fagg and Lane, 1979; Johnston, Allan, Kennedy and Twitchin, 1979 ; Krogsgaard-Larsen, 1980.

isonipecotic acid, kojic amine (Yarbrough, 1979) and THIP, they are all much more effective neuronal inhibitors than GABA.

The results for one member of this group, piperidine-4-sulphonate (P4S) are compared with GABA in Figures 18 and 19. As well as P4S all other members of this group depolarized group Ia terminations, although these effects were generally of slower onset and recovery than those produced by GABA. Muscimol, dihydromuscimol, P4S, 3-aminopropanesulphonic acid (3APS) and isoguvacine were more potent than GABA and depolarized terminals on which GABA was ineffective (to a maximum of 80 nA). Isonipecotic acid, kojic amine and THIP were approximately equipotent with GABA although these compounds, and other members of this group, usually maintained plateau levels of depolarization even at values exceeding those at which the depolarization by GABA faded or reversed to an increase in threshold. BMC readily reduced or abolished the depolarizing effect of these compounds. In Figure 18B, the electrophoretic administration of BMC elevated the threshold of the terminal by 22%, abolished the sustained depolarization by P4S and the initial transient depolarization by GABA and reduced PAD from 35 to 21% (not illustrated in Figure 18B). A similar effect of BMC on the action of P4S and PAD is illustrated in Figure 19. Recovery of PAD and the depolarization by P4S occurred 5 min after terminating the BMC current with the terminal illustrated in Figure 18B.

Figure 18A shows the slow onset and recovery (over 5 min) of a 20% decrease in threshold of a Ia termination by muscimol which was accompanied by a reduction of PAD and the conversion

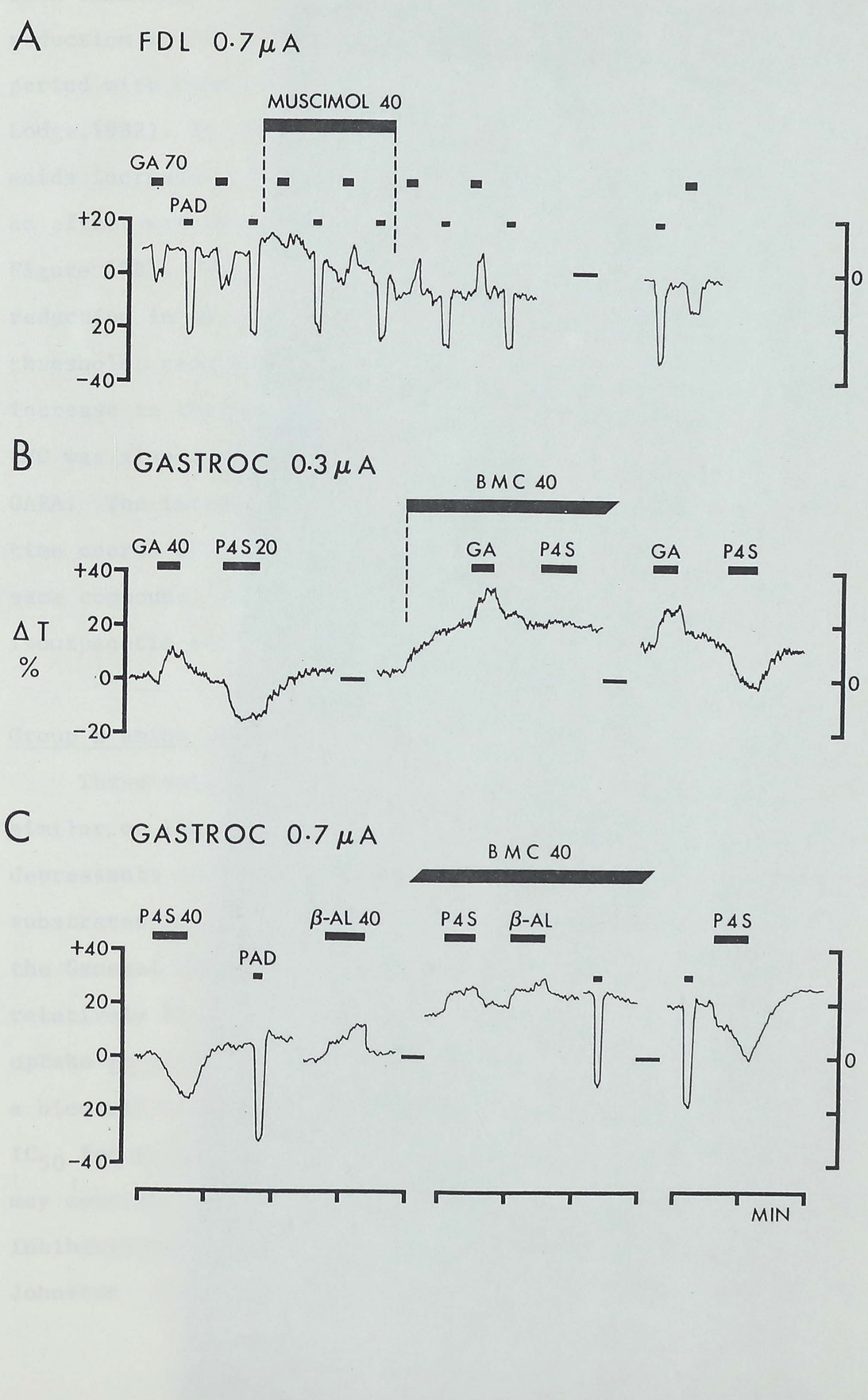


FIGURE 18 Changes in the threshold (10 Hz stimulation, 50% response) of electrically stimulated Ia terminations produced by microelectrophoretically administered amino acids for the times indicated by the horizontal bars (current, nA): GABA (GA), piperidine-4-sulphonate (P4S), β-alanine (β-AL). Primary afferent depolarization (PAD) was produced by tetanic stimulation of the posterior biceps-semitendinosus nerve (four volleys, 2.5T, 320 Hz) 30 ms prior to the testing pulse. Ordinates: percentage change in control threshold value, as indicated in μA for each termination. Abscissae: time in minutes. A flexor digitorum longus termination (FDL, blocking factor 3); before, during and after muscimol (40 nA). The gap in the record is of 5 min duration. B. Gastrocnemius termination (blocking factor 3); before, during and 5 min after bicuculline methochloride (BMC, 40 nA). C. Gastrocnemius termination (blocking factor 3.8); before, during and 7 min after BMC (40 nA).

of a GABA-induced decrease in threshold to an increase. A reduction in PAD often occurred during the prolonged recovery period with compounds such as muscimol (see also Curtis and Lodge,1982). In the absence of BMC, none of this group of amino acids increased the threshold of Ia terminations even when such an effect was the predominant or sole effect of GABA (see Figure 18B). However, as shown in Figure 18C, BMC converted a reduction in threshold by P4S into a small increase in threshold, reduced PAD, but was ineffective against the increase in threshold by a group C amino acid (β-alanine). BMC was also ineffective against increases in threshold by GABA. The increase in threshold by P4S had a relatively rapid time course compared with that of the depolarization by the same compound. Similar results were seen with muscimol, 3APS, isonipecotic acid and THIP.

Group B amino acids

These amino acids are structural analogues of GABA having similar or lower potencies (apart from DL-2-chloro-GABA) as depressants of neuronal firing. These compounds are also substrates for the GABA uptake mechanisms (see Section d of the General Introduction) being effective antagonists at relatively low concentrations (see Table 2, column 3) of GABA uptake *in vitro*. Imidazole-4-acetic acid is also included as a bicuculline-sensitive GABA-mimetic, since, although the IC_{50} for uptake is high, a high capacity uptake process may contribute to the rapid recovery of neuronal firing after inhibition by this amino acid (Curtis, Duggan, Felix and Johnston, 1971).

These compounds had variable effects. In many cases (see Figure 19C for DL-2-hydroxy-GABA) there was an initial transient decrease in threshold followed by an increase. With some terminations, however, the only effect was an increase, which like the group A amino acids, was more readily apparent in the presence of electrophoretic BMC. During reductions in threshold by these compounds PAD was again reduced. This effect was particularly obvious with 4-methyl-aminobutyric acid which also reduced depolarizations by GABA and P4S. In contrast, PAD was unaffected when amino acids of this group elevated the threshold of Ia terminations, suggesting that such elevation was not associated with a conductance increase shunting the underlying PAD.

The results with D-nipecotic acid were variable: this compound depolarized nine of 19 terminations, increased the threshold of six and had no effect on four. The depolarizing action was relatively weak compared to GABA and did not reduce PAD. When depolarization of two terminations by D-nipecotic acid was blocked by BMC, a clear increase in threshold was observed. When ejected from dilute solution D-nipecotic acid enhanced the effect of GABA, increasing the depolarization of 14 Ia terminations, having no effect on one and reducing the depolarization of another. However, PAD was not enhanced by D-nipecotic acid ejected from either dilute or concentrated solutions.

In Figure 19A DL-2-hydroxy-GABA produced minimal initial reduction in threshold compared with GABA, a reduction in threshold being the only response to P4S. In four of 18 terminations so tested, however, DL-2-hydroxy-GABA produced an

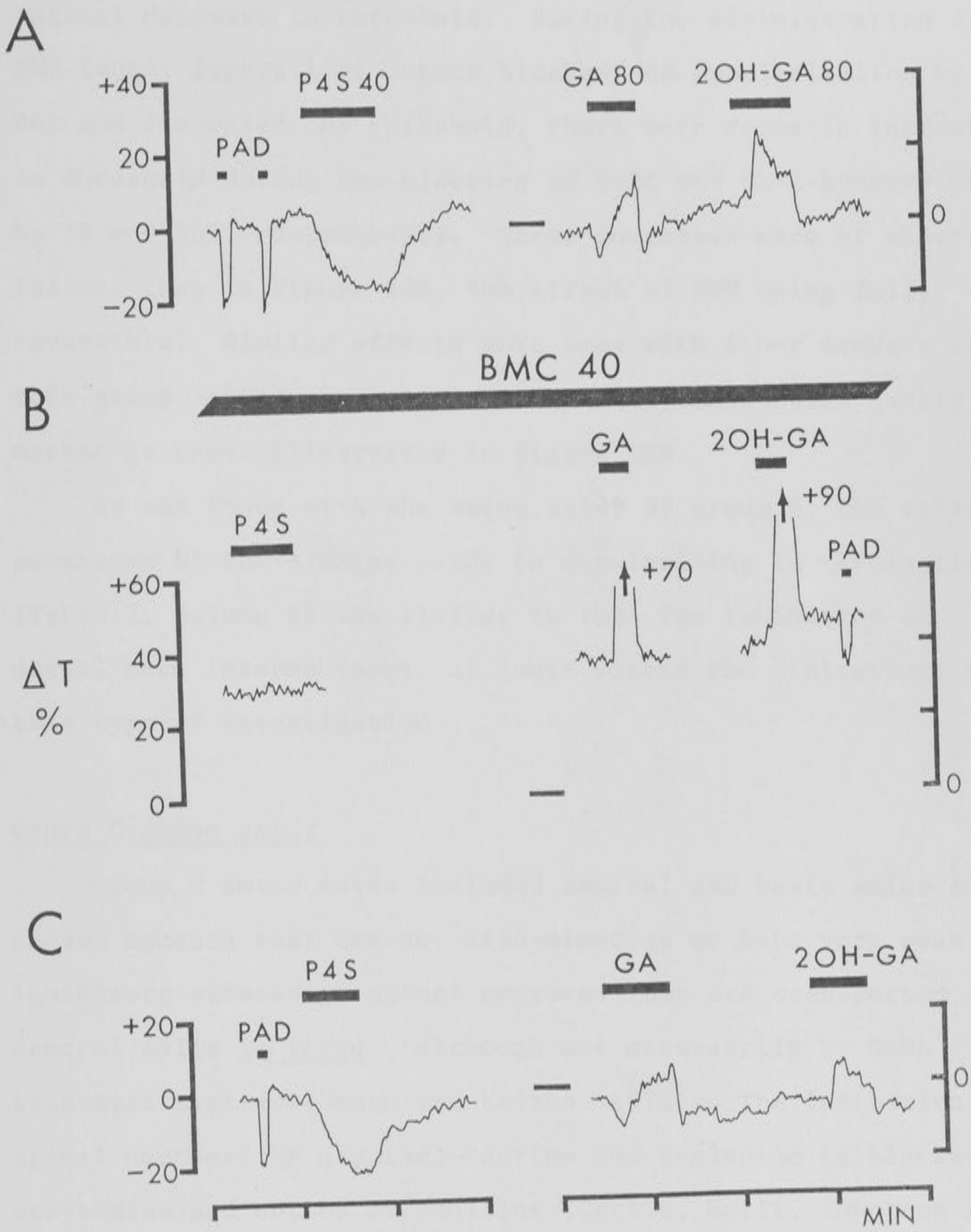


FIGURE 19 The actions of P4S, GABA and DL-2-hydroxy-GABA (2-OH-GA). A. before: B. during BMC (40 nA) the records are truncated at the arrows, thresholds increased by 70 and 90% for GA and 2-OH-GA, respectively; C. 12 min after BMC. Ordinates: and abscissae as for Figure 18.

initial decrease in threshold. During the administration of BMC (40nA: Figure 19B), which blocked the depolarization by P4S and increased the threshold, there were dramatic increases in threshold during the ejection of GABA and DL-2-hydroxy-GABA by 70 and 90%, respectively. These increases were of shorter latency than in Figure 19A, the effect of BMC being fully reversible. Similar effects were seen with other members of this group, although the increases in threshold were rarely as marked as those illustrated in Figure 19B.

As was found with the amino acids of group A, the relative potencies of these amino acids in depolarizing Ia terminations (Table 2, column 6) was similar to that for inhibition of dorsal horn interneurones, at least within the limitations of this type of investigation.

Group C amino acids

Group C amino acids included neutral and basic amino acids chosen because they are not GABA-mimetics or have very weak inhibitory effects on spinal neurones, but are transported into central cells *in vitro* , although not necessarily by GABA transport systems (Cohen and Lajtha, 1972). The inhibition of spinal neurones by glycine, taurine and β-alanine is blocked by strychnine and not by bicuculline (Curtis, Hösli, Johnston and Johnston, 1968; Curtis, Duggan, Felix and Johnston, 1971). Consistent inhibition has not been reported for DL-ornithine, L-lysine, L-arginine or L-2,3-diaminopropionic acid, and the inhibitory effects of L-histidine (Lodge, 1979), L-2,4-diamino-butyric acid (Curtis and Watkins, 1960) and 2,2-dimethyl-β-alanine (Curtis, Game and Lodge, 1976) are too weak to enable

an adequate study to be made of the effects of either bicuculline or strychnine. Both glycine and β-alanine are taken up by cat spinal tissues *in vitro* (Lodge, Johnston and Stephanson, 1978), and, although similar techniques indicate that taurine is not readily taken up by any transport system in these tissues (Lodge, Johnston, Curtis and Brand, 1977), the rapid recovery of neuronal inhibition after microelectrophoretic taurine (Curtis, Duggan, Felix and Johnston, 1971; Curtis, Game and Lodge, 1976) suggests that an effective uptake process may be present *in vivo* (Karczmarek and Davison, 1972; Hruska, Padjen, Bressler and Yamamura, 1978).

Figure 18C illustrates an increase in termination threshold by β-alanine (40 nA) by 5%. This change, like that produced by GABA, was unaffected by BMC. β-Alanine increased the threshold of seven of nine terminations and reduced the threshold (5-10%) of two. These depolarizations were of slow onset and recovery, did not affect PAD, and in one where BMC was tested, the depolarization was converted into a brief increase in threshold.

Glycine reduced the threshold of only two of 45 terminations, all of which were depolarized by GABA. This effect, although weaker and of slower onset and recovery than that of GABA, was accompanied by a reduced PAD. The threshold of the majority of the other terminations were unaffected by glycine (60-80 nA), but those of 14 were increased by 5-10%, an effect consistent with the earlier report of Sverdlov and Kozhechkin, 1975. Taurine, ejected as a cation with currents of 40-80 nA, also increased the threshold of all seven terminations by 5-20% but had no effect on PAD.

FIGURE 20 The actions of GABA, L-histidine (HIST), 2,2-dimethyl-β-alanine (DMβA), L-2,3-diamino-propionic acid (DAPA) and L-lysine (LYS). A. Comparison of the effects of GABA and HIST. B. Before, during and 5 min after BMC (40 nA). C. Reduction in threshold by GABA compared with increases by other acids. Ordinates and abscissae as for Figure 18.

In Figure 20C the effect of 2,2-dimethyl-β-alanine(DMβA), L-2,3-diaminopropionic acid (DAPA) and L-lysine (LYS) were compared with GABA on a gastrocnemius Ia termination. Whereas the threshold was reduced by GABA, the threshold was increased by the other compounds. Similar results were obtained with L-ornithine, L-arginine and L-2,4-diaminobutyric acid, none of these amino acids reducing the threshold of Ia terminations.

L-Histidine was ejected near 59 terminations and increased the threshold of all but five which were unaffected. This increase in threshold, which was maximal in 10-30 s, was independent of the direction of the change produced by GABA. The increase did not significantly alter PAD and was not affected by BMC. The effect of histidine on a number of terminations is illustrated in Figures 20, 21 and 22.

In Figure 20A this termination was depolarized by GABA (5 nA) which was followed by an increase in threshold at higher currents (shown for 40 nA). The 40% increase in threshold by L-histidine was not preceded by a decrease, and PAD was increased from 25 to 28% in contrast to the decrease observed during the administration of GABA. These increases in threshold by L-histidine and GABA recovered rapidly. With the second termination (Figure 20B) concentrations of BMC adequate to reduce PAD, and reversibly convert an unmaintained reduction in threshold by GABA (40 nA) into an increase in threshold, had no effect on the action of L-histidine.

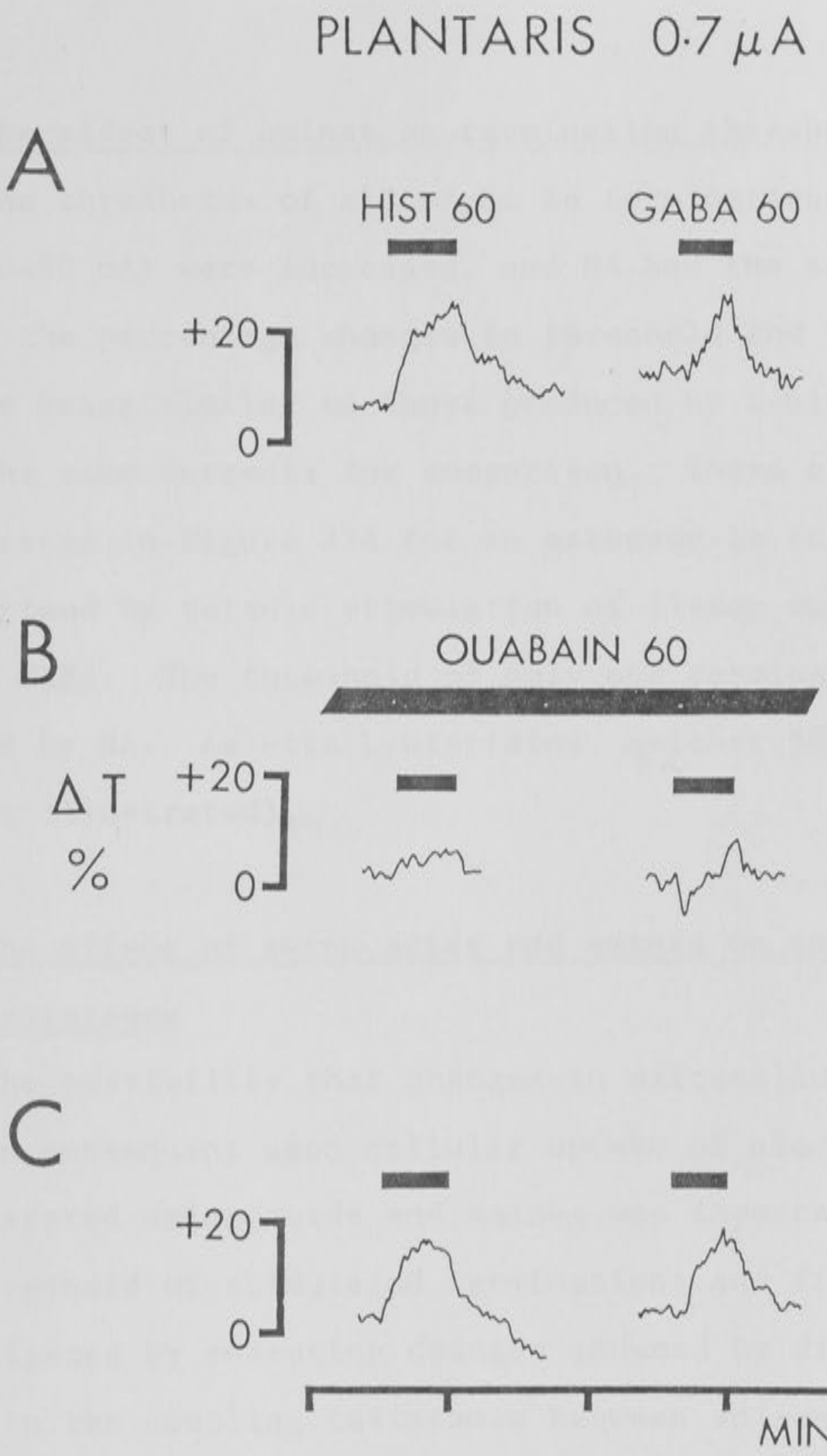


FIGURE 21 The effect of L-histidine (60 nA) and GABA (60 nA) on a plantaris Ia termination threshold. A. Before; B. 6 min during ouabain (1 mM in 150 mM NaCl, 60 nA for 7.4 min); C. 5 min after ouabain. Ordinates and abscissae as for Figure 18.

(b) The effect of amines on termination thresholds

The thresholds of all eight Ia terminations tested with 5HT (40-90 nA) were increased, and NA had the same effect on seven, the percentage changes in threshold and their time courses being similar to those produced by L-histidine ejected with the same currents for comparison. These effects are illustrated in Figure 22A for an extensor Ia termination depolarized by tetanic stimulation of flexor muscle afferents, K^+ and GABA. The threshold of only one termination was lowered by NA. As with L-histidine, neither 5HT nor NA reduced PAD(not illustrated).

(c) The effect of amino acids and amines on coupling resistance

The possibility that changes in extracellular ion concentration consequent upon cellular uptake of electrophoretically administered amino acids and amines was important in modifying the threshold of stimulated terminations and fibres was investigated by measuring changes induced by different amino acids in the coupling resistance between adjacent barrels of seven barrel micropipettes (see Methods). Measurements of the extracellular coupling resistance were made during the administration of amino acids using electrophoretic currents (20-80 nA) similar to those producing changes in termination threshold. Most investigations were carried out using L-histidine since this amino acid reliably increased termination thresholds, this effect not being complicated by preceding or concomitant depolarization.

Coupling resistances were generally increased, sometimes by as much as 50%, during the ejection of L-histidine with currents of 20-80 nA in the vicinity of motoneurones. As illustrated in Figure 23A and B, the ejection of L-histidine (60 nA) increased the coupling resistance by about 15%. The time course of this change was similar to that for changes in termination threshold produced by this amino acid, with recovery occurring very rapidly. In addition, coupling resistances (Table 2, column 7) were increased by L-arginine, 4-aminovaleric acid, β-alanine and imidazole acetic acid. Although increases by these compounds were of similar magnitude, those produced by GABA, glycine, taurine, THIP, kojic amine, DABA, P4S and muscimol were usually smaller and of longer latency.

When ejected with similar currents to those that increased the threshold of Ia terminations, NA (Figure 24) and 5HT also increased the coupling resistance between the barrels of seven barrel micropipettes by as much as 50%, the time course of the change also being similar to that of the alteration in termination threshold.

(d) Ouabain: effects on amino acid- and amine-induced alteration in termination theshold and coupling resistance

Ouabain, a cardiac glycoside known to inhibit the Na^+-dependant uptake of amino acids and amines(Iversen, 1975; Martin, 1976) was used in this study since many of the relatively specific inhibitors of the cellular uptake of particular amino acids and structural analogues are transported by the same sodium dependent mechanism, and hence would not be

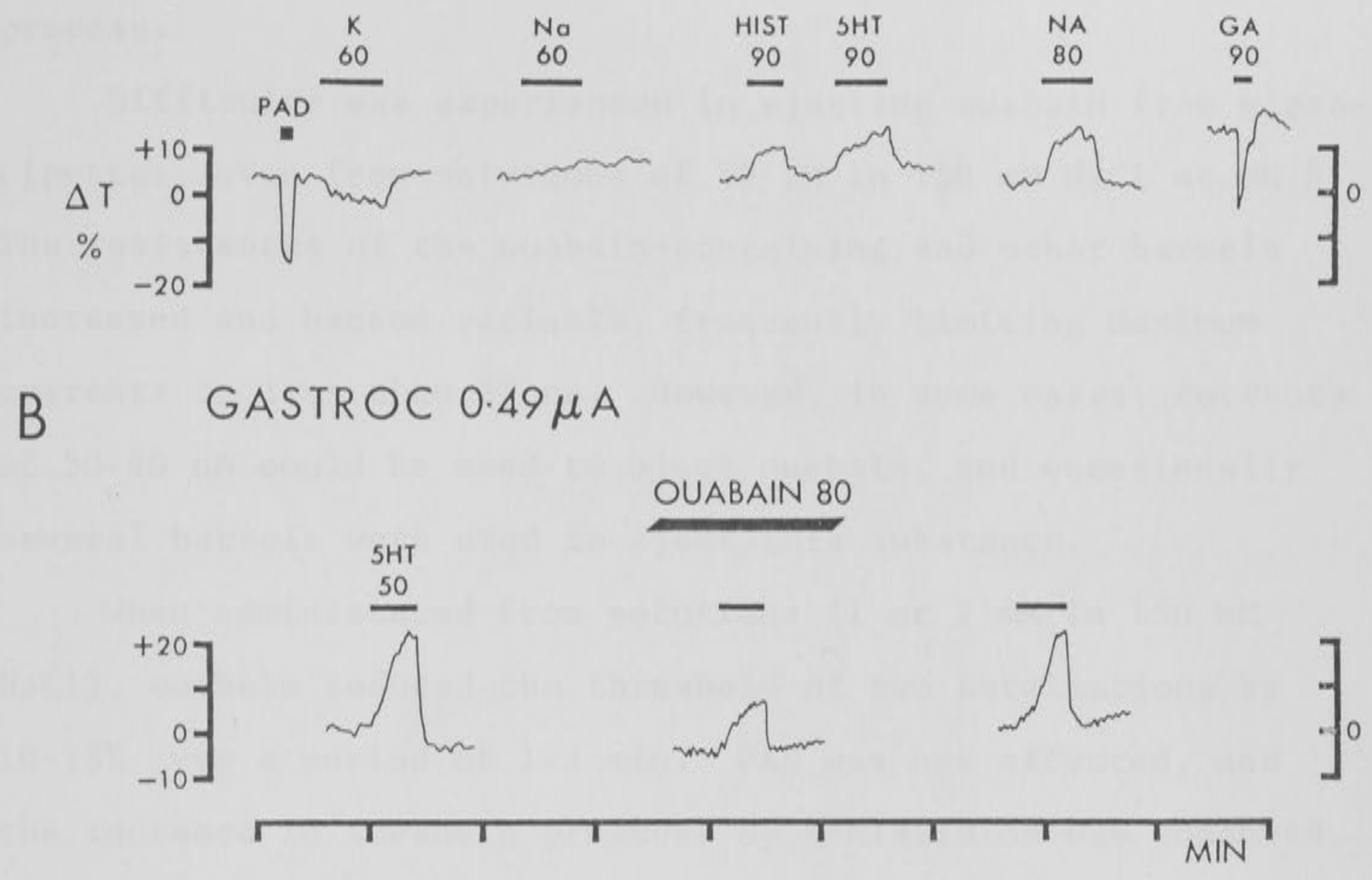


FIGURE 22 A. Changes in the threshold of an extensor Ia termination produced by electrophoretically-administered potassium (K), sodium (Na), L-histidine (HIST), 5-hydroxytryptamine (5HT), noradrenaline (NA) and GABA and by synaptically-generated PAD. Ejection of compounds is indicated by the horizontal bars and currents (nA). B. The effect of 5HT (50 nA) on termination threshold before, 5 min during ouabain (1 mM in 150 mM NaCl, 80 nA), 6 min after ouabain. Ordinates and abscissae as for Figure 18.

suitable for studying events resulting from the transport process.

Difficulty was experienced in ejecting ouabain from micropipettes, even from solutions of 10 µM in 150 mM NaCl at pH 5 The resistances of the ouabain-containing and other barrels increased and became variable, frequently limiting maximum currents to less than 30 nA. However, in some cases, currents of 50-60 nA could be used to eject ouabain, and occasionally several barrels were used to eject this substance.

When administered from solutions (1 or 2 mM in 150 mM NaCl), ouabain reduced the threshold of two terminations by 10-15% over a period of 1-2 min. PAD was not affected, and the increase in theshold produced by L-histidine was enhanced, all effects recovering within several minutes of terminating the ejection of ouabain. With two other terminations, ouabain had no effect on either the threshold or the actions of L-histidine, but with seven others the thresholds were increased with a similar time course to the reduction in threshold produced by ouabain. Figure 21A-C shows the reduction and recovery of the threshold increases by L-histidine and GABA by ouabain 60 nA (1 mM in 150 mM NaCl). It is also apparent in this Figure that a depolarizing effect of GABA was revealed or enhanced during ouabain, presumably by the reduction of the associated increase in threshold. In Figure 22B, the effect of a similar concentration of ouabain on the increase in termination threshold by 5HT is illustrated.

The effects of ouabain on the increase in coupling resistances produced by L-histidine were also complex. A reversible reduction of the L-histidine-induced increase in coupling resistance was observed more frequently when the

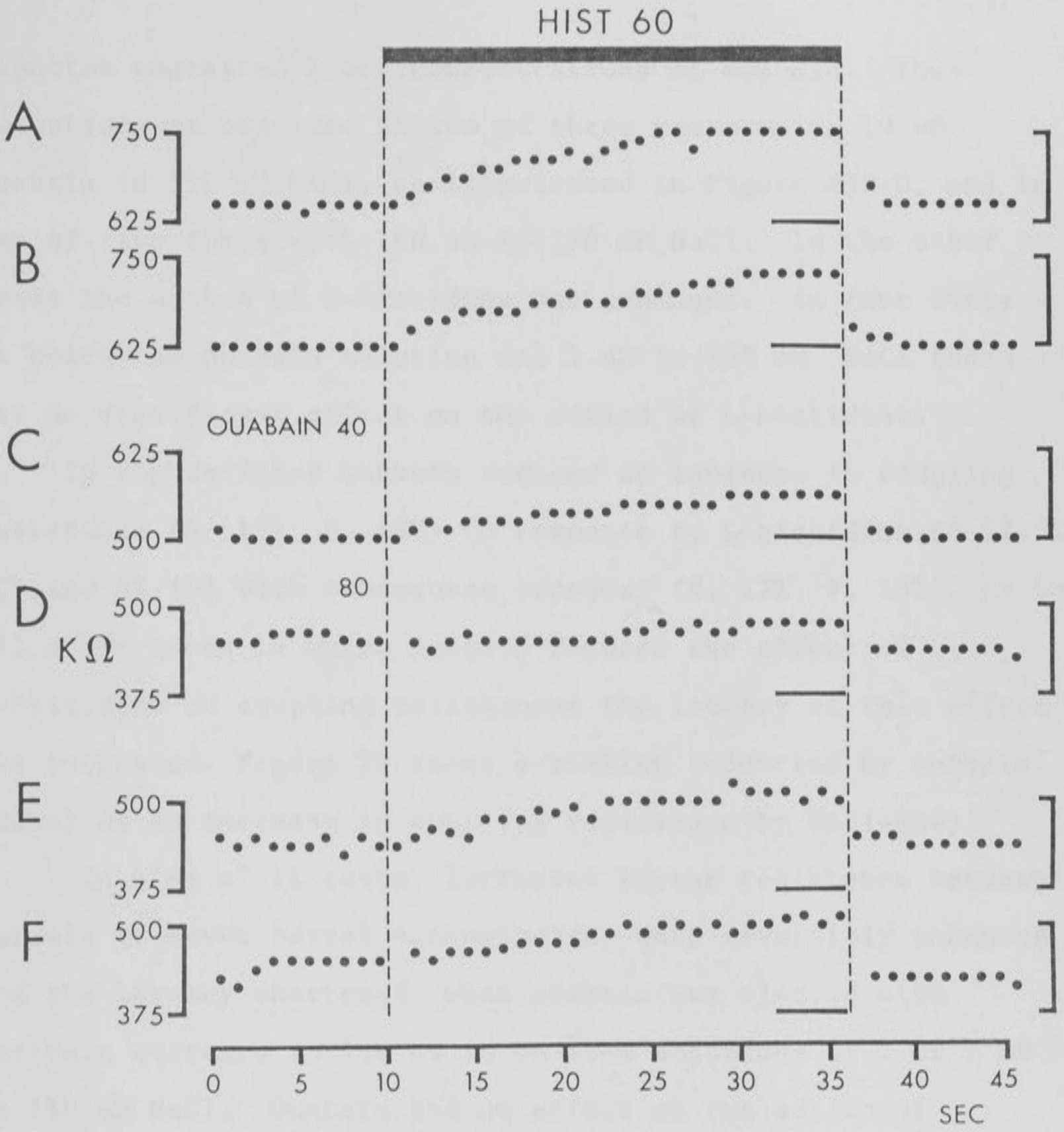


FIGURE 23 The effect of L-histidine (60 nA, A-F) on the coupling resistance between the central and a peripheral barrel of a seven barrel micropipette, the tip of which was near motoneurones, measured as described in methods; before (A,B), during (C, 40 nA for 2.6 mins; D, 80 nA for 2.5 min) and after (E, 30 s; F, 3 min) administration of ouabain (10 µM in 0,15 M NaCl). Ordinates: KΩ; Abscissae: time in seconds.

pipettes contained lower concentrations of ouabain. Thus reduction was observed in two of three tests using 10 μM ouabain in 150 mM NaCl, as illustrated in Figure 23C-D, and in two of five tests with 100 μM in 150 mM NaCl. In the other tests the action of L-histidine was enhanced. In four tests in which the ouabain solution was 1 μM in 150 mM NaCl there was no significant effect on the action of L-histidine.

In Figure 23A-F ouabain reduced an increase in coupling resistance (A, 15%; B, 16%) in response to L-histidine to 12.5% (C) and 8% (D) with subsequent recovery (E, 17%; F, 15%). As in all other tests in which ouabain reduced the effect of L-histidine on coupling resistances the latency of this effect was increased. Figure 24 shows a similar reduction by ouabain (30nA) of an increase in coupling resistance by NA (40nA).

In nine of 11 tests, increases in the resistance between barrels of seven barrel micropipettes were reversibly enhanced and the latency shortened, when ouabain was ejected with cationic currents as low as 10 nA from solutions of 1 or 2 mM in 150 mM NaCl. Ouabain had no effect on the action of L-histidine in one test, and reversibly decreased it in the other. As with the effect of ouabain on histidine-induced changes in termination threshold, these effects had a latency of 1-2 min, and recovery was very rapid, often within 1 min. Ouabain, which alone had no consistent effects on coupling resistances, similarly enhanced increases in coupling resistance produced by P4S (one test) and imidazole-4-acetic acid (four tests).

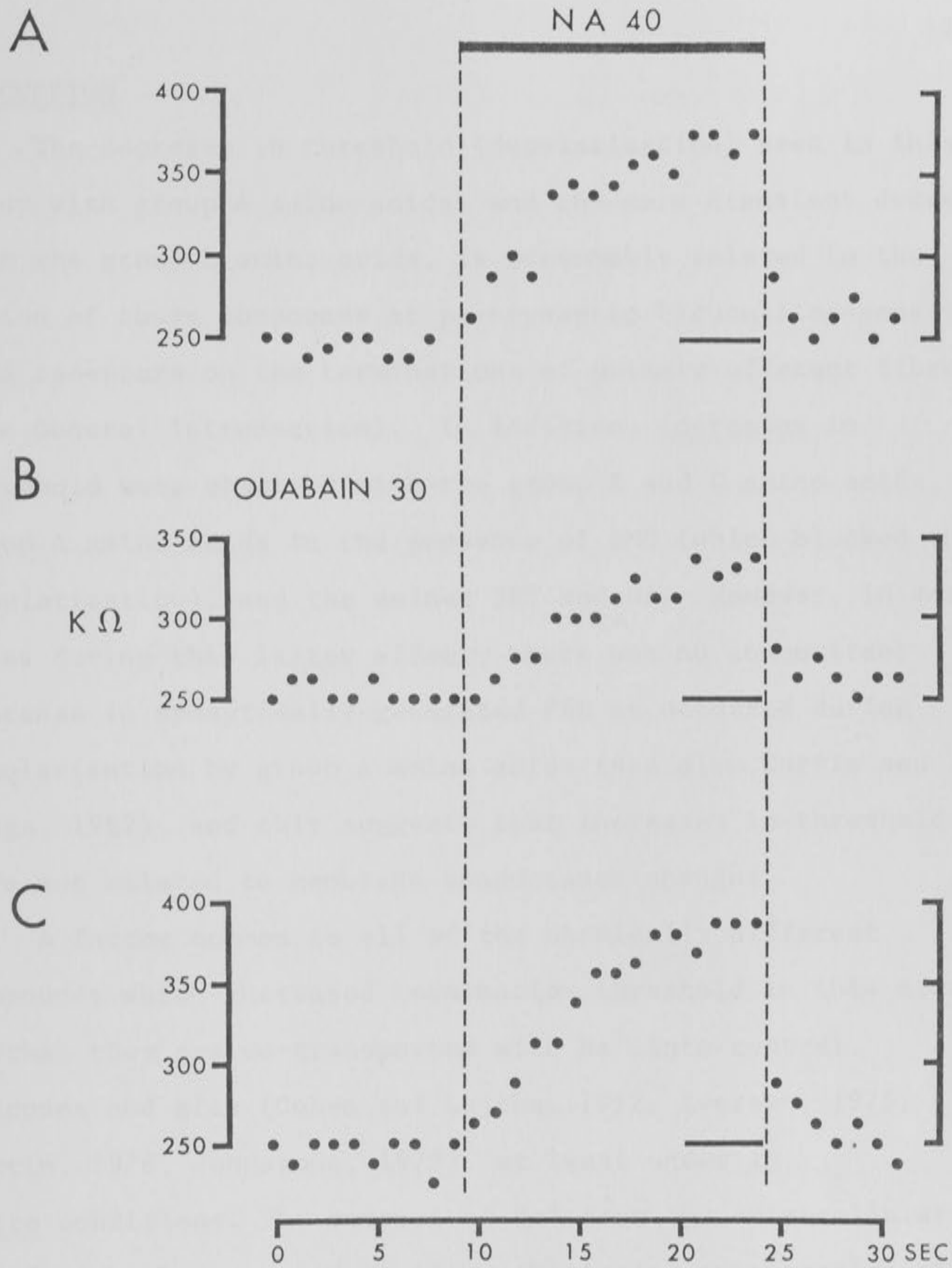


FIGURE 24 Changes in the coupling resistance produced by NA (40 nA) ejected for the period between the vertical broken lines. A: before; B, 2 min during ouabain (100 µM in 150 mM NaCl, 30 nA); C: 1 min after ouabain. Ordinates and abscissae as for Figure 23.

DISCUSSION

The decrease in threshold (depolarization) seen in this study with group A amino acids, and the more transient decrease with the group B amino acids, is presumably related to the action of these compounds at postsynaptic bicuculline-sensitive GABA receptors on the terminations of primary afferent fibres (see General Introduction). In addition, increases in threshold were observed with the group B and C amino acids, the group A amino acids in the presence of BMC (which blocked the depolarization), and the amines 5HT and NA. However, in most cases during this latter effect, there was no concomitant decrease in synaptically-generated PAD as occurred during depolarization by group A amino acids (see also Curtis and Lodge, 1982), and this suggests that increases in threshold were not related to membrane conductance changes.

A factor common to all of the chemically different compounds which increased termination threshold in this study is that they are co-transported with Na^+ into central neurones and glia (Cohen and Lajtha, 1972; Iversen, 1975; Martin, 1976; Johnstone, 1979), at least under in vitro conditions. The removal of Na^+ from the extracellular fluid might be expected to alter the ionic concentration of this solution, with subsequent alteration in the threshold level for stimulation of the afferent terminal under investigation. Alterations in the coupling resistances between adjacent barrels of seven barrel micropipettes which were measured in this investigation suggest that there were decreases in extracellular ion concentration (probably more than 15-50 mM) in the immediate vicinity of the orifices of the micropipettes from which GABA and other compounds were ejected

for many seconds. With particular relevance to this observation, Rudomin, Engberg and Jimenez (1981) reported changes in the amount of current flowing through excitability testing microelectrodes during the ejection of GABA (50-100 nA) from an adjacent barrel, an effect reduced when the electrode projected beyond the orifice of the GABA barrel. Such effects were never seen with similar currents ejecting NaCl or when electrodes were tested in isotonic saline. These observations are also consistent with changes in coupling resistance being largely determined by alteration in the properties of the medium close to the orifice of the amino acid barrel, although it should be pointed out that such changes may not necessarily originate in, or be restricted to, this region but could also be as a result of redistribution of ions to adjacent sites.

Increases in coupling resistance, should they represent alterations in the ionic concentration should also occur in areas other than regions of afferent terminations. Measurements of excitability of Ia fibres (blocking factors >6) within the spinal gray matter dorsal to motoneurones showed that GABA, L-histidine and L-arginine increased the threshold with time courses and extents similar to those observed for their actions on terminations. In contrast, P4S had either no effect on, or decreased the threshold of fibres, although this effect was of very slow onset and recovery compared to the time course of the reduction of termination threshold by P4S, and may have resulted from the diffusion (since P4S is a poor substrate for amino acid uptake systems; Krogsgaard-Larsen,

Falch, Shousboe, Curtis and Lodge, 1980) of P4S to the terminal regions of the Ia fibre being stimulated. Such a result is important since it suggests that accurate localization of the stimulating microelectrode is necessary if the effect of electrophoretically-ejected compounds on primary afferent terminals is to be investigated. In recent papers (Carstens, Klumpp, Randić and Zimmermann, 1981; Jeftinija, Semba and Randić, 1981; Randić, Carstens, Zimmermann and Klumpp, 1982), sites for stimulation and microelectrophoretic ejection were within spinal laminae I-III where the threshold for afferent fibres was lowest and PAD could be detected as a reduction in threshold in response to stimulation of peripheral afferents. Such criteria, however, may be insufficient to verify that terminal portions of A and C fibres were being studied. Sites of lowest threshold may not necessarily be terminals, especially of non-myelinated axons, and as is the case for Ia fibres, the electrotonic spread of synaptically-generated PAD can be detected as a decreased threshold in non-terminal regions. In addition, the measurement of blocking factors, lower values of which can be expected for non-myelinated structures than for the parent myelinated afferent fibre (see Curtis and Lodge, 1982) is not applicable to the terminals of non-myelinated afferents. Thus in these recent studies (Carstens, Klumpp, Randić and Zimmermann, 1981; Jeftinija, Semba and Randić, 1981; Randić, Carstens, Zimmermann and Klumpp, 1982), it is possible that impulses recorded antidromically in peripheral nerves in response to relatively large intraspinal stimuli may have been initiated some distance from both terminals and the tips of the stimulating microelectrode within the spinal cord.

The effects of ouabain on both the increases in termination threshold and the coupling resistances in response to both amino acids and amines were complex. Since ouabain would not be ionized in solution, its ejection would be predominantly electro-osmotic, and the local concentrations close to the pipette orifices would be determined largely by the concentration of ouabain within the micropipettes. Levels of 1 or 2 mM considerably exceed those used under in vitro conditions (μM) to inhibit amino acid uptake. Hence uptake processes close to the pipette orifices would be totally inhibited, such that concentrations of L-histidine would probably be much higher, and in a larger volume of tissue beyond the immediate vicinity of the pipette orifices, than in the absence of ouabain. The enhancement of the coupling resistance increase presumably reflects a much greater disturbance of ion concentrations as L-histidine diffuses beyond the volume of tissue influenced by ouabain. However, the reduction of amino acid and amine-induced increases in termination threshold and coupling resistance by ouabain is consistent with the concept that this compound blocks the NA^+-coupled uptake of amino acids and amines from the extracellular medium.

Other factors are obviously also important during experiments using excitability testing. For example, the spatial relationship of the electrode to the terminal is unoubtedly important. As was mentioned in Methods slow drifts in termination threshold could be reversed by quite small adjustments of the position of the microelectrode. Thus, a

relatively sudden and localized reduction in the concentration of the major extracellular cation might alter the geometry of the extracellular space, particularly if this depletion of extracellular cations produces a concomitant efflux of water from this region. The duration of such changes would presumably be dependent upon ionic pump acitivity to restore the correct ionic balance between the intra- and extracellular compartments.

Thus the results of this study suggest that the reduction in threshold by group A amino acids results from a GABA-like change in membrane conductance producing a depolarization of the terminal portion of the afferent fibre. However, the increase in threshold of afferent terminations by amino acids and amines appears not to be related to direct effects on membrane conductances, but to changes in ion concentrations in the extracellular fluid resulting from Na^+-coupled uptake of these amino acids and amines into neurones and glia. The direction, extent and time course of any direct and indirect effects (dual actions seen with group B amino acids and group A amino acids in the presence of BMC) on termination threshold produced by a microelectrophoretically-administered compound would thus be a complex function of the rate of ejection, the efficacy at depolarizing GABA receptors on terminations, the effects on nearby neurones, and the rate of removal by uptake or other inactivating processes, together with the rate at which disturbed levels of extracellular ion concentrations are restored by processes which maintain "normal" levels. Thus, in the absence of direct intracellular recording of membrane potential and conductance changes in afferent

terminations, the nature of the "hyperpolarization" by GABA proposed to account for the increases in thresholds of rubrospinal and reticulospinal terminals in the studies of Rudomin, Engberg, Jankowska and Jimenez (1980) and Rudomin, Engberg and Jimenez (1981) requires further investigation. Such increases may well have resulted from alterations in the extracellular ion environment rather than from direct hyperpolarization by GABA of these terminals. If this be so, the observations of these investigators may be quite irrelevant to a rôle of synaptically-released GABA in controlling the release of transmitter from the terminals of these descending pathways.

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